Simultaneous HPLC Analysis of Asiaticoside and α-Mangostin in Nanoethosomal Carriers

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Received: September 12, 2025
Accepted: November 10, 2025

DOI: 10.22146/ijc.111150

Abstract: *The development of robust analytical methods is essential for quality control of nanoethosomal formulations containing asiaticoside and α-mangostin, bioactive compounds with anti-inflammatory and antioxidant properties. Simultaneous quantification within nanocarriers is challenging and requires validated techniques. This study established a precise HPLC method, in accordance with ICH Q2(R2) guidelines, for the concurrent determination of both compounds. The technique demonstrated excellent linearity (R^2 > 0.999), precision (%RSD < 2%), and accuracy (95–105% recovery). The nanoethosomal system also achieved high encapsulation efficiencies of 94.76% for asiaticoside and 99.27% for α-mangostin. This validated method provides a reliable tool for standardizing these complex nano-formulations, supporting their further development and clinical translation.*

Keywords: *HPLC; validation; asiaticoside; α-mangostin; nanoethosome*

■ INTRODUCTION

The increasing interest in herbal-based pharmaceutical formulations has driven the development of novel delivery systems designed to enhance the bioavailability and therapeutic efficacy of bioactive natural compounds. Asiaticoside and α-mangostin, both derived from traditional medicinal flora, exhibit diverse pharmacological properties. Asiaticoside, a triterpenoid saponin primarily extracted from *Centella asiatica*, is recognized for its skin-regenerative, anti-inflammatory, and wound-healing effects [1]. In parallel, α-mangostin, a xanthone sourced from *Garcinia mangostana* (mangosteen), has demonstrated substantial antibacterial and antioxidant capabilities [2]. The potential synergistic effects of combining these compounds may offer enhanced therapeutic benefits in the treatment of skin disorders such as acne and wound dermatitis. The practical necessity for a simultaneous quantification method stems from the growing interest in developing co-formulated topical products that leverage these synergistic effects. Although a commercial product with this exact combination is not yet widely marketed, robust analytical methods are a prerequisite for the quality control (QC) of such innovative formulations during their development, manufacturing, and stability testing. Nonetheless, their clinical utility is frequently hindered by poor stability and suboptimal bioavailability.

In this context, nanoethosomes, a lipid-based nanocarrier system, present a promising strategy to mitigate these limitations. Composed primarily of phospholipids, ethanol (20–50%), and water [3],

nanoethosomes facilitate the encapsulation of asiaticoside and α-mangostin. This system has emerged as a superior delivery platform, significantly enhancing the bioavailability of bioactive compounds [4]. The encapsulation within these nanocarriers serves a dual purpose: it improves the stability of the compounds and enhances their penetration through the skin layers for topical applications [5]. This has critical implications for the development of topical formulations, as encapsulating compounds such as asiaticoside and α-mangostin not only preserves their therapeutic integrity but also substantially enhances their overall therapeutic effectiveness. To assure the quality, safety, and efficacy of such formulations, it is paramount to develop a robust analytical method capable of simultaneously quantifying both compounds in nanoethosomal suspensions. Recent advancements in high-performance liquid chromatography (HPLC) have significantly enhanced the analysis of multiple natural compounds in complex matrices, making it an indispensable tool for such tasks.

HPLC is widely recognized for its efficacy in quantifying natural products, owing to its high sensitivity, precision, and accuracy [6]. According to recent studies by Chahomchuen et al. [7], HPLC methods ensure precise and reliable quantification of plant-derived compounds, enhancing the overall reliability of pharmaceutical formulations. Moreover, advancements in method validation, as explored by Anjani et al. [8], underscore the importance of confirming the accuracy and reliability of HPLC methods, ensuring that quantification results align with actual values within acceptable tolerances. These developments underscore the need to validate the analytical process in accordance with ICH Q2(R2) guidelines to ensure robustness and reproducibility. However, the simultaneous determination of asiaticoside and α-mangostin poses analytical challenges due to their distinct chemical structures and physicochemical properties. Muchtaridi et al. [9], demonstrated the effectiveness of HPLC in isolating target compounds from matrix interferences, a challenge also encountered in our analysis of nanoethosomal formulations. This makes HPLC an indispensable tool for ensuring the purity and concentration of bioactive compounds in complex herbal formulations. Thus, establishing a validated HPLC method for the concurrent quantification of these two constituents is essential for standardizing and controlling the quality of nanoethosomal formulations.

While HPLC methods for asiaticoside [10] or α-mangostin [11] individually exist, publications on the simultaneous quantification of both compounds within a complex nanoethosomal matrix range from limited to none, as of the writing of this manuscript. This represents a significant analytical challenge due to their vastly different polarities and the interference from lipid-based matrix components. Therefore, the novelty of this work lies in the development and validation of the first stability-indicating HPLC method, following Analytical Quality by Design (AQbD) principles, for the simultaneous analysis of asiaticoside and α-mangostin in nanoethosomes. This method is crucial for accurately determining encapsulation efficiency and ensuring quality control in dual-compound nanoformulations.

This study seeks to develop and validate a straightforward, accurate, and precise HPLC method for the simultaneous determination of asiaticoside and α-mangostin in nanoethosomal suspensions. The validation process will adhere to the ICH Q2(R2) guidelines [12], assessing critical parameters such as linearity and range, limits of detection (LOD) and quantitation (LOQ), selectivity, precision, and accuracy. The anticipated outcomes are expected to bolster the quality control efforts for nanoethosomal formulations, thereby facilitating their progression towards further development and clinical application.

■ EXPERIMENTAL SECTION

Materials

Asiaticoside (≥ 95%, HPLC grade) and α-mangostin (≥ 90%, HPLC grade) were obtained from Markherb, Indonesia. Standardized extracts of *C. asiatica* and *G. mangostana* were sourced from the Department of Medical Pharmacy, Faculty of Medicine, Universitas Indonesia. Reagents, including ethanol, sodium hydroxide, potassium dihydrogen phosphate, dichloromethane, methanol, and acetonitrile were procured from Merck, Germany. Phosphatidylcholine

(Phospholipon 80G) and ultrapure water were generously provided by Lipoid, Germany.

Instrumentation

Chromatographic analysis was performed using a Shimadzu SPD-20A HPLC system (Shimadzu, Japan) with a UV-vis detector, utilizing a C18 column (250 mm × 4.6 mm, 5 µm, YMC-Triart, Germany). A Shimadzu UV-1800 UV-vis spectrophotometer (Shimadzu, Japan) was used for wavelength scanning. Nanoethosomes were prepared using a Büchi R-300 rotary vacuum evaporator (Büchi, Switzerland), with dispersion assisted by a probe sonicator (QSonica, Newton, USA). pH was measured with a pH meter (Eutech Instrument pH 510, Singapore).

Procedure

Preparation and characterization of nanoethosomes

Nanoethosomes were synthesized utilizing the thin-film hydration technique. Phospholipids were initially dissolved in dichloromethane, while the extracts of *C. asiatica* and *G. mangostana* were solubilized in ethanol. The lipid extract was subsequently evaporated in a rotary vacuum evaporator at 40 °C and 150 rpm, yielding a thin lipid film. The film was then purged with nitrogen and stored at 4 °C for up to 24 h. For hydration, an ethanol-phosphate buffer solution (pH 7.4) was used in conjunction with glass beads, and the process was carried out under gradual rotation at 50–250 rpm at 37 °C. The resultant nanoethosomes suspension was sonicated at 25% amplitude for 5 min to reduce vesicle size. The resulting nanoethosomes were immediately characterized to ensure batch quality and suitability for subsequent analytical validation. The fully characterized nanoethosomal suspension was stored at 4 °C for subsequent applications.

Characterization of the nanoethosomes vehicle

The physicochemical properties of the nanoethosomes were characterized using dynamic light scattering (DLS) and electrophoretic light scattering. Vesicle size, polydispersity index (PDI), and zeta potential were measured with a Zetasizer (Malvern PANalytical, UK). Prior to analysis, the samples were diluted to 0.5% w/v with deionized water and stirred for 3 min to ensure homogeneity. All measurements were performed in triplicate, and the results are expressed as mean ± standard deviation.

Nanoethosomal encapsulation efficiency

The percentage of encapsulation efficiency (%EE) was determined using the indirect centrifugation method. A total of 10 mL of nanoethosomal suspension was centrifuged at 10,000 rpm for 1 h, resulting in a pellet and a supernatant. The supernatant was diluted with methanol, and the concentration of the free (unentrapped) active substance (C_s) was analyzed using the validated HPLC method. The total drug concentration (C_t) was determined by diluting a separate aliquot of the non-centrifuged nanoethosomal suspension with methanol after vigorous vortex mixing. The %EE was calculated using Eq. (1).

$$\%EE = \frac{C_t - C_s}{C_t} \times 100\% \quad (1)$$

HPLC conditions

The determination of α-mangostin and asiaticoside was conducted using a reversed-phase HPLC system, specifically a Shimadzu SPD-20A (Shimadzu, Japan), equipped with a UV-vis detector. The analytical separation employed a YMC-Triart C18 column (YMC, Germany), 12 nm with dimensions of 250 × 4.6 mm and a particle size of 5 µm, maintained at a controlled temperature of 25 °C throughout the experimental runs. The mobile phase was delivered at a consistent flow rate of 1 mL/min, and an injection volume of 20 µL was utilized to ensure optimal detection and quantification of the compounds of interest.

Preparation of stock solution and calibration solution

A stock solution of asiaticoside and α-mangostin was prepared by dissolving 10 mg of each standard in 10 mL of 70% ethanol, resulting in individual concentrations of 1,000 µg/mL. Subsequently, a series of calibration solutions was prepared by further diluting the stock solution, yielding concentrations of 5, 10, 25, 50, 100, and 150 µg/mL for both compounds. This methodical approach ensures accurate assessment of compound concentrations for analytical purposes.

Determination of analytical wavelength

Optimization of the analytical wavelength for the HPLC system was performed using a UV spectrophotometer (Shimadzu, Japan). Absorption spectra were systematically acquired for 1 μg/mL α-mangostin and 25 μg/mL asiaticoside in ethanol over 200–400 nm. The resultant absorption curves for both compounds overlapped effectively, and the optimal wavelength was determined by analyzing the absorbance profiles of each compound. This approach ensured precise detection within the HPLC system, thereby enhancing analytical performance.

Optimization of the mobile phase

Optimization of the mobile phase composition was performed by injecting a mixture containing 50 μg/mL solutions of asiaticoside and α-mangostin into the HPLC system. Variations in the mobile phase were achieved by utilizing different combinations of methanol or acetonitrile with water in volumetric ratios of 70:30, 80:20, and 90:10 (v/v). The determination of the optimal mobile phase was based on the evaluation of peak shapes for asiaticoside and α-mangostin observed in the chromatograms and on the resolution between the respective peaks. This approach enabled a comprehensive assessment of chromatographic performance, facilitating the identification of conditions that yield the highest quality separation of the target compounds.

System suitability test

In this study, a standard mixed solution containing asiaticoside and α-mangostin at 50 μg/mL was prepared and subsequently analyzed under optimized chromatographic conditions. The parameters assessed during this evaluation included retention time (t_R), peak area, number of theoretical plates (N), resolution (R_s), and tailing factor (T_f). These metrics are critical for assessing the efficiency and reliability of the chromatographic separation achieved in this analysis.

Validation

Linearity and range. The analytical procedure commenced with the filtration of six distinct concentrations of the mixed standard solutions through a 0.45 μm RC syringe filter, before subsequent analysis. Each solution was analyzed in triplicate (n = 3). Calibration curves for asiaticoside and α-mangostin were generated by plotting peak area versus concentration. To evaluate the linearity of the method, the linear equations, coefficients of determination (R^2), and the defined test ranges were meticulously recorded.

LOD and LOQ. The determination of the LOD and LOQ was conducted using standard solutions, with a sample size of n = 3. The LOD and LOQ were computed utilizing the standard deviation of the peak area of the standard solutions (σ) in conjunction with the slope of the calibration curve (S). This approach ensures the reliability and precision of the analytical method employed.

Selectivity. The selectivity of the analytical method was confirmed by comparing the retention times of asiaticoside and α-mangostin in the nanoethosomal formulation with those of their respective standards. The absence of interfering peaks within the retention windows of the target analytes in the blank matrix suggests that the method is suitably specific for the simultaneous determination of asiaticoside and α-mangostin. Furthermore, the selectivity was further reinforced by evaluating the chromatographic profile of the blank nanoethosomal formulation, which showed no discernible peaks at the retention times of the asiaticoside and α-mangostin standards. This absence of peaks serves as an additional indicator of the method's selectivity.

Accuracy. The accuracy of the analytical method was evaluated utilizing the spike recovery approach. Given this methodology, three distinct concentrations of standards (40, 50, and 60 μg/mL) were systematically introduced into extracts of *C. asiatica* and *G. mangostana* incorporated within nanoethosome formulations. These formulations contained known concentrations of the bioactive compounds asiaticoside and α-mangostin. Following spiking, the samples were filtered through a 0.45 μm RC syringe filter to remove particulate matter. The filtered samples were then analyzed in triplicate (n = 3). The results were quantified as percentage recovery, serving as an indicator of the method's accuracy.

Precision. Precision was rigorously assessed utilizing three distinct concentrations of mixed standard solutions: 40, 50, and 60 µg/mL. Before analysis, the solutions were filtered through a 0.45 µm RC syringe filter to remove impurities. Each concentration was analyzed in triplicate (n = 3). The repeatability of the measurements was evaluated by calculating the percentage relative standard deviation (%RSD) of results obtained on the same day. Furthermore, intermediate precision was determined by calculating %RSD from measurements conducted across three separate days.

■ RESULTS AND DISCUSSION

Determination of Analytical Wavelength

Selecting an appropriate detection wavelength was critical for the simultaneous analysis of asiaticoside and α-mangostin, given their distinct UV-vis profiles. As shown in Fig. 1, asiaticoside and α-mangostin exhibited maximum absorbances at 206 and 212 nm, respectively. A wavelength of 210 nm was established as an optimal compromise, providing robust sensitivity for both compounds within a single run. This strategic selection was pivotal for enhancing the signal-to-noise ratio and ensuring precise quantification, which is often challenging in complex nanoethosomal matrices where excipient interference can be significant [13-14]. The stable baseline and absence of interfering peaks at this wavelength further confirm the method's selectivity, making it suitably robust for analyzing lipid-based formulations where matrix effects are a common concern [14-15]. Consequently, 210 nm was selected as the analytical wavelength, providing enhanced sensitivity for both compounds while effectively minimizing potential interference from formulation excipients, as evidenced by the overlaid spectra shown in Fig. 1.

Optimization of Mobile Phase Composition

The composition of the mobile phase was systematically optimized to enhance the separation efficiency of asiaticoside and α-mangostin during HPLC analysis. The primary objectives included achieving well-resolved, non-overlapping peaks, ensuring appropriate retention times, and achieving sufficient sensitivity for

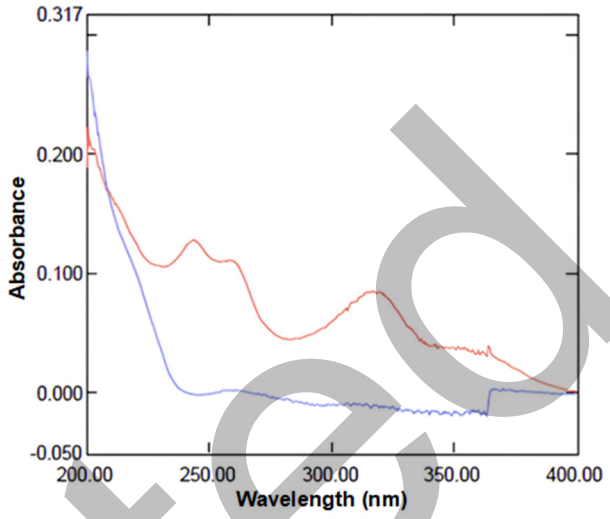


Fig 1. Overlaid UV-vis spectra of α-mangostin (blue line, 1 µg/mL) and asiaticoside (red line, 25 µg/mL) in ethanol, showing maximum absorbances at 212 nm and 206 nm, respectively. The dashed line indicates the selected detection wavelength of 210 nm for simultaneous HPLC analysis

precise quantification. Additionally, efforts were made to minimize both analysis time and solvent consumption, thereby improving the overall analytical efficiency [16].

Initial experimental trials using methanol-water mixtures at 70:30, 80:20, and 90:10 ratios revealed limitations in analyte separation. In all tested ratios, the asiaticoside peak was detected at approximately 3 min but co-eluted with the solvent peak, significantly impeding its quantification. Notably, in the 70:30 ratio, the α-mangostin peak remained undetectable. However, the α-mangostin peaks were distinctly separated in the 80:20 and 90:10 ratios, exhibiting retention times of 16.0 and 13.6 min, respectively. Despite this improvement, the insufficient separation of asiaticoside from the solvent at these ratios rendered them unsuitable for further analytical applications.

In light of these observations, subsequent evaluations were conducted using acetonitrile-water mixtures at 70:30 and 80:20 ratios. The 70:30 ratio resulted in extended retention times for both asiaticoside (16.7 min) and α-mangostin (26.1 min). Conversely, the 80:20 ratio yielded shorter retention times, with asiaticoside detected at 9.2 min and α-

mangostin at 12.3 min. This ratio also exhibited sharper and more symmetrical peaks, indicative of improved resolution. Furthermore, the increase in peak areas at this ratio suggested enhanced analyte detection and heightened sensitivity. Consequently, acetonitrile-water (80:20) was determined to be the optimal mobile phase for the simultaneous separation of these two compounds.

The optimization process confirmed acetonitrile-water (80:20) as the optimal mobile phase, a choice directly informed by the distinct polarities of the target analytes. The strong elution strength and low viscosity of acetonitrile were critical for achieving rapid, efficient separation, effectively eluting the highly hydrophobic α-mangostin while maintaining a sharp peak shape for the more polar asiaticoside. This resulted in an excellent resolution ($R_s > 2$) and a total run time of under 15 min, enhancing the method's throughput for routine analysis [17]. The high organic proportion was particularly beneficial for solubilizing α-mangostin within the mobile phase, thereby preventing on-column precipitation and ensuring robust quantification—a common challenge when analyzing high-loading nano-formulations of poorly soluble actives [18]. Consequently, this mobile phase selection was not merely a theoretical preference but a practical necessity to ensure the method's reliability for its intended application in quality control.

System Suitability Test

A system suitability test was conducted to ensure the chromatographic system was appropriate for the analysis of asiaticoside and α-mangostin. The evaluation parameters comprised retention time (t_R), peak area, number of theoretical plates (N), resolution (R_s), and tailing factor (T_f). As emphasized by Rajput et al., these parameters are crucial for determining the robustness and efficiency of chromatographic methods, particularly when analyzing multiple active pharmaceutical ingredients (APIs) in complex formulations [19]. This assessment was performed in accordance with the guidelines outlined in ICH Q2(R2) [10], ensuring compliance with established methodologies for analytical validation, the acceptance criteria were defined as follows: retention times should be consistent with minimal variability, %RSD of peak areas should not exceed 2.0%, N should be ≥ 2000 to indicate efficient column performance, R_s should be ≥ 2.0 to ensure adequate peak separation, and T_f should be ≤ 2.0 to confirm peak symmetry.

All evaluated parameters consistently aligned with the predetermined criteria, with %RSD values for peak areas remaining below 2%. This outcome underscores the stability, precision, and efficiency of the chromatographic system for the simultaneous determination of asiaticoside and α-mangostin. These results align with those of Aggarwal et al., who emphasized the importance of robust chromatographic performance in pharmaceutical analysis for achieving consistent and reliable results [13]. Comprehensive results from the system suitability test are presented in Table 1.

Method Validation

The developed HPLC method was rigorously validated in accordance with ICH Q2(R2) guidelines to establish its reliability for the simultaneous quantification of asiaticoside and α-mangostin in nanoethosomal formulations. The validation comprehensively assessed critical parameters, including selectivity, linearity, accuracy, precision, and sensitivity (LOD and LOQ), as summarized in Table 2.

Selectivity

The selectivity of the method was demonstrated by comparing chromatograms of the standard substances,

Table 1. System suitability test results

Parameter	Asiaticoside	%RSD	α-Mangostin	%RSD	Acceptance criteria
Retention Time (t_R) (min)	9.175 ± 0.016	0.178	11.557 ± 0.031	0.264	Consistent with minimal variability
Area	24,387.200 ± 439.034	1.800	3,790,898.600 ± 72,753.217	1.919	≤ 2.0%
Tailing factor (T_f)	1.616 ± 0.024	1.512	0.908 ± 0.011	1.262	≥ 2.0
Number of theoretical plates (N)	11,091.200 ± 215.120	1.940	7,241.600 ± 104.438	1.442	≥ 2000
Resolution (R_s)	2.761 ± 0.040	1.450	2.132 ± 0.025	1.157	≤ 2.0

The value is presented in mean ± standard deviation (n = 5)

Table 2. Results of validation of analysis methods

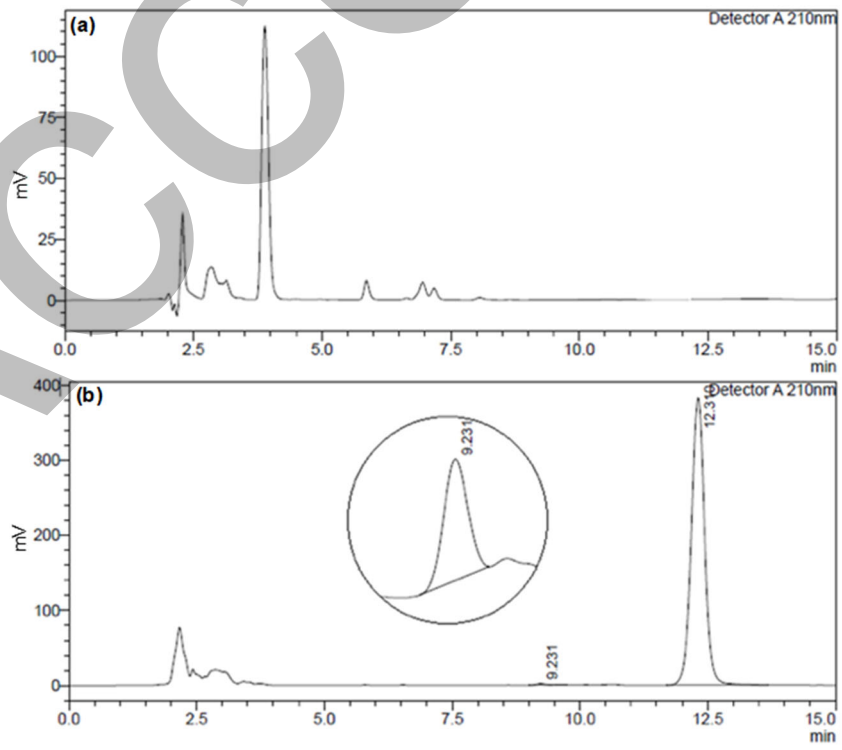
Parameter	α-Mangostin	Asiaticoside
Calibration curve regression equation	y = 73408.545x – 69961.7	y = 237.5904x – 134.122
Calibration curve correlation coefficient (r)	0.9999	0.9999
LOD (µg/mL)	2.5	2.4
LOQ (µg/mL)	8.4	8.1
Recovery (n=3) (%)	97.0–104.7	101.9–103.4
Precision repeatability (n = 3) (%)	0.25–0.61	0.51–0.98
Intermediate precision (n = 3) (%)	0.14–1.80	0.8–1.9

a blank nanoethosomal formulation (placebo), and the finished product. The analysis showed exemplary selectivity, with both α-mangostin and asiaticoside distinctly separated from potential interfering substances. Critically, no interfering peaks from the placebo were observed at the retention times of 9.1 min for asiaticoside and 12.3 min for α-mangostin. The chromatographic profiles of the excipients further corroborated the absence of peaks at the specified retention times, thereby ensuring the precise and reliable identification and quantification of the analytes in the presence of the formulation excipients.

These findings align with prior studies in which validated HPLC methods demonstrated clear separation and high selectivity for α-mangostin, with retention times ranging from 9.6 to 12.3 min, and confirmed the absence of interference from matrix components [20]. Additionally, the robustness of the method's selectivity was further validated in studies using high-performance thin-layer chromatography (HPTLC), in which α-mangostin was effectively separated from other phytochemicals. This outcome attests to the method's applicability and reliability in analyzing complex matrices [21]. Representative chromatograms are illustrated in Fig. 2.

Linearity

The method demonstrated excellent linearity (R^2 = 0.9999) for both asiaticoside and α-mangostin over a concentration range of 5–150 µg/mL (Fig. 3). This wide



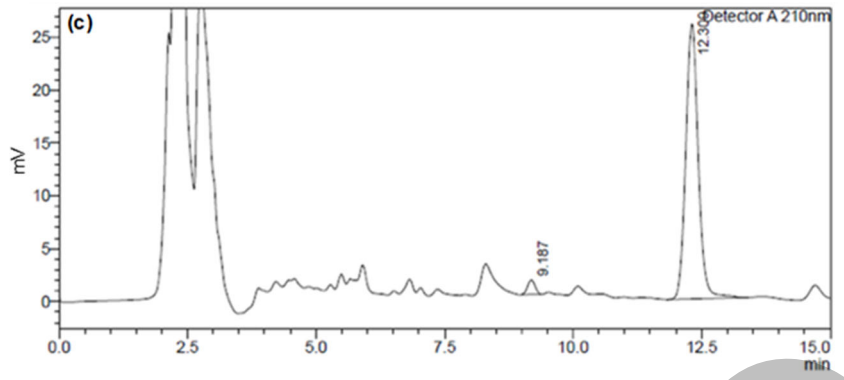


Fig 2. Representative HPLC chromatograms demonstrating selectivity: (a) Blank nanoethosomal formulation (excipients), showing no interference at the analyte retention times; (b) Standard mixture of asiaticoside (t_R = 9.1 min) and α-mangostin (t_R = 12.3 min); (c) Nanoethosomal formulation loaded with both compounds. Although the peak height of asiaticoside is lower due to its inherent UV absorptivity, its LOD is comparable to that of α-mangostin due to excellent baseline stability

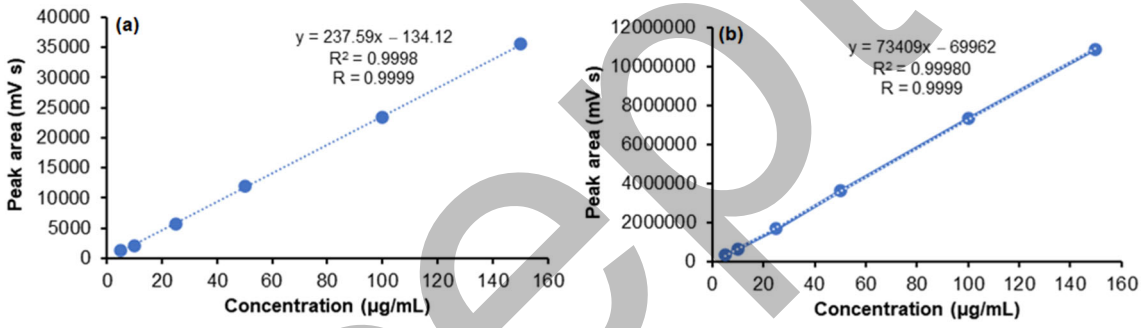


Fig 3. Standard calibration curves for (a) asiaticoside (y = 237.59x − 134.12, R^2 = 0.9999) and (b) α-mangostin (y = 73408.55x − 69961.7, R^2 = 0.9999) over the concentration range of 5–150 µg/mL

linear dynamic range is particularly crucial for the intended application, as it readily accommodates the varying analyte concentrations encountered during formulation development and quality control—from the low concentrations used in encapsulation efficiency studies to the high concentrations in the total drug content assay. The consistency of this result with prior studies on α-mangostin [22-23] further reinforces the robustness of reversed-phase HPLC for quantifying this compound. More importantly, the exceptional linearity across this range ensures reliable quantification without the need for sample dilution, thereby streamlining the analytical workflow for routine quality control of nanoethosomal batches.

LOD and LOQ

The LOD refers to the minimum concentration of an analyte that can be reliably detected. At the same time, the LOQ denotes the lowest concentration that can be measured with acceptable accuracy and precision. For asiaticoside, the determined LOD and LOQ were 2.4 and 8.1 µg/mL, respectively. For α-mangostin, the LOD and LOQ were 2.5 and 8.4 µg/mL, respectively. Although the peak height of asiaticoside in the chromatogram (Fig. 2(b)) appears lower than that of α-mangostin due to its inherent UV absorptivity, the calculated LOD values for both compounds were comparable. This can be attributed to the excellent baseline stability and low noise, which allowed for a favorable signal-to-noise ratio for asiaticoside even at low concentrations. These findings underscore the method's high sensitivity for detecting and precisely quantifying low concentrations of both analytes. Recent studies have reported similar LOD and LOQ values, highlighting RP-HPLC's sensitivity and suitability for analyzing low-

concentration compounds in pharmaceutical formulations [14,24]. Previous investigations have reported even lower LOD and LOQ values for α-mangostin, with measurements as low as 0.06 and 0.17 μg/mL, respectively. These results corroborate the efficacy of reverse-phase high-performance liquid chromatography (RP-HPLC) as a highly sensitive analytical technique, making it suitable for routine analysis and quality control of mangostin compounds in herbal matrices.

Accuracy and precision

The evaluation of accuracy and precision for the quantitative determination of asiaticoside and α-mangostin in nanoethosomal formulations was conducted concurrently to ensure the reliability of the analytical method employed. Accuracy was determined through recovery tests, in which known concentrations of the analytes were added to the sample matrix. The resulting recovery values ranged from 95 to 105%, thereby satisfying the acceptance criteria established for chemical and pharmaceutical analysis [25]. This range demonstrates the method's ability to yield results closely aligned with actual values. Additionally, the robustness of accuracy was further substantiated through recovery assessments conducted over multiple days, which yielded consistent findings well within the acceptable limits.

Precision was quantified as %RSD and examined for both repeatability and intermediate precision. Standard solutions at concentrations of 40, 50, and 60 μg/mL were systematically analyzed in triplicate. The resultant %RSD values for both asiaticoside and α-mangostin were below 2%, indicating high precision and reproducibility across same-day and inter-day analyses. A comprehensive summary of the combined results, including accuracy and precision, is presented in Table 3.

The physicochemical characterization and encapsulation efficiency data collectively demonstrate the successful development of a high-quality nanoethosomal formulation. The system exhibited excellent colloidal properties, with a particle size of 429.22 nm, a homogeneous distribution (PDI = 0.47), and a high negative zeta potential of −39.63 mV, confirming good physical stability, which is essential for reliable analytical sampling. Critically, these properties directly contributed to outstanding encapsulation efficiencies of 94.76% for asiaticoside and 99.27% for α-mangostin (Table 4). This demonstrates the system's exceptional capability to co-load and retain two chemically diverse actives, a fundamental prerequisite for achieving a synergistic therapeutic effect. The near-quantitative encapsulation of the highly hydrophobic α-mangostin is particularly noteworthy, as it confirms the efficacy of the lipid-based ethosomal matrix in solubilizing and stabilizing challenging compounds. Consequently, these integrated results validate the nanoethosome not merely as a carrier, but as an efficient and stable delivery platform tailored for dual-compound therapy [26].

This study is dedicated to the development and validation of a comprehensive analytical method for the simultaneous quantification of asiaticoside and α-mangostin within a nanoethosomal drug delivery system. Both compounds are recognized for their significant therapeutic properties. However, their distinct chemical characteristics, particularly in terms of polarity and stability, present considerable challenges for concurrent analysis. The nanoethosomal formulation, comprising

Table 3. Accuracy and precision of asiaticoside and α-mangostin analysis

Compounds	Sample conc. (μg/mL)	Spike conc. (μg/mL)	Precision RSD (%)		Accuracy
			Intraday	Interday	Recovery (%) ± SD
Asiaticoside	62.145	40.342	0.973	1.114	103.428 ± 1.152
		51.438	0.981	1.942	101.920 ± 1.979
		59.662	0.513	0.884	102.803 ± 0.909
α-Mangostin	7.601	40.342	0.614	0.147	103.968 ± 0.153
		51.438	1.422	1.757	97.062 ± 1.706
		59.662	0.547	1.834	104.754 ± 1.921

The value is presented in mean ± standard deviation (n = 3)

Table 4. Calculation of the encapsulation efficiency of nanoethosomes

Compounds	Total drug content (mg/mL)	Free drug (mg/mL)	Encapsulation efficiency (%)	mean ± SD
Asiaticoside	3.036	0.160	94.720	94.637 ± 0.213
	2.764	0.155	94.395	
	2.754	0.143	94.796	
α-Mangostin	3.931	0.029	99.256	99.267 ± 0.015
	3.876	0.029	99.262	
	4.127	0.030	99.284	

The value is presented in mean ± standard deviation (n = 3)

phospholipids, ethanol, and water, forms a complex matrix that may interfere with chromatographic methods. Such matrix components can adversely affect baseline stability, lead to peak overlap, and diminish detection sensitivity—issues that are similarly underscored in the review by Agrawal et al. [27], which discusses lipid-based vesicular systems.

The HPLC method established in this research offers several analytical advantages, facilitating its implementation for both routine and advanced evaluations of nanoethosomal formulations. The technique successfully achieves high resolution between asiaticoside and α-mangostin, despite their divergent polarities and retention times, thereby ensuring clear separation and precise quantification. Furthermore, the method exhibits low LOD and LOQ, enabling reliable measurement of trace levels of both compounds. The criteria that are essential for conducting stability studies and release profiling. As reported by Kaviani [28], different methods for determining LOD and LOQ values highlight the variability and importance of selecting appropriate methods for accurate drug quantification, further reinforcing the reliability of this technique for your formulations.

To ensure the stability of the analytes, meticulous control of column temperature and protection of samples from light during injection were implemented, effectively preventing degradation of these sensitive compounds. Notably, the method was developed using an AQbD approach, which facilitates systematic parameter optimization and enhances robustness and reproducibility under stress conditions. These findings align with those reported by Usgaonkar et al. [26], who emphasized the advantages of AQbD-based HPLC method development for stability-indicating assays.

The developed HPLC methodology presents several notable advantages over previously reported analytical techniques, thereby enhancing its practicality and efficiency. This approach enables the simultaneous detection of two chemically distinct analytes, asiaticoside and α-mangostin, within a single chromatographic run, thereby significantly reducing analysis time and solvent consumption. This capability is particularly advantageous in high-throughput environments, such as formulation screening and quality control assessments. Additionally, the method demonstrates exceptional selectivity within a complex nanoethosomal matrix, effectively mitigating interference from lipids and ethanol. These interferences are commonly encountered challenges in conventional analytical methods, underscoring the robustness of the developed HPLC technique [28]. Furthermore, the methodology demonstrates strong performance even under various stress conditions, encompassing acidic, basic, oxidative, and photolytic environments. It adheres strictly to the ICH Q1A(R2) and Q2(R1) guidelines. This stability-indicating capability is essential for affirming the method's reliability throughout the stages of product development, long-term storage, and regulatory assessment.

The efficacy of nanoethosomes as drug delivery carriers has been extensively documented, particularly for their capacity to enhance drug permeation, reduce particle size, and increase bioavailability [29]. However, a notable limitation persists in the existing literature: many investigations into nanoethosomes fail to provide

thorough validation of the analytical methods employed. The current study aims to bridge this gap by not only formulating a novel nanoethosomal system but also by establishing a rigorously validated analytical methodology for quantifying drug content and assessing performance metrics within these delivery systems.

Notably, as articulated by Su et al. [30], conventional analytical methodologies continue to encounter significant challenges in assessing the *in vivo* behavior and long-term stability of nano-formulations. The incorporation of advanced techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and bio-imaging, has the potential to enhance the applicability of these methods for pharmacokinetic and biodistribution studies. Such advancements would yield profound insights into the interactions and behaviors of nanoethosomal drugs within biological systems. Nevertheless, persistent issues, including instability under physiological conditions, insufficient *in vivo* characterization, and the absence of standardized methodologies, pose substantial impediments to the accurate prediction of nanoformulation performance and the successful translation into clinical applications [31-32].

The validated HPLC method offers substantial practical utility for the pharmaceutical development of nanoethosomal co-formulations of asiaticoside and α-mangostin. Its simplicity, accuracy, and precision make it directly applicable to routine quality control (QC), including drug content assays in final products and encapsulation efficiency monitoring during formulation optimization. The method's selectivity, confirmed through forced degradation studies, establishes its stability-indicating capability, crucial for monitoring product stability and detecting degradation under stress conditions [33]. This aligns with established practices, in which validated HPLC methods are recognized as essential tools for ensuring the consistency and stability of complex lipid-based nanoformulations [34]. The critical importance of such robust analytical techniques is further underscored by recent advances in encapsulating α-mangostin within various nanocarriers (e.g., nanofibers, nanosponges, lipid nanoparticles), where they are indispensable for verifying drug loading, ensuring physicochemical stability, and profiling release kinetics—all fundamental to the quality assurance of modern nano-pharmaceuticals [35-36].

To the best of our knowledge, this is the first reported HPLC method for the simultaneous quantification of asiaticoside and α-mangostin. A comparison with existing individual methods [10-11] shows that our combined method offers a comparable performance in terms of linearity, accuracy, and precision, while providing the distinct advantage of analyzing both compounds in a single, time-efficient run (analysis time < 15 min), thereby reducing solvent consumption and increasing analytical throughput for co-formulated products.

■ CONCLUSION

A reliable HPLC method was successfully developed and validated for the simultaneous determination of asiaticoside and α-mangostin in nanoethosome suspensions, using a C18 column (250 × 4.6 mm, 5 µm), acetonitrile-water (80:20) mobile phase, 1.0 mL/min flow rate, 20 µL injection volume, and wavelength detection at 210 nm. The method exhibited excellent linearity, sensitivity, selectivity, accuracy, and precision, demonstrating its suitability for analyzing complex nanoethosomal matrices. The nanoethosome formulation showed high encapsulation efficiencies (94.76% for asiaticoside and 99.27% for α-mangostin), confirming its effectiveness in stabilizing and delivering the compounds. These findings provide a solid basis for quality control and standardization of nanoethosomal formulations, with further studies suggested on long-term stability, *in vitro* and *in vivo* performance, and method optimization.

■ ACKNOWLEDGMENTS

The authors gratefully thank Universitas Indonesia for the financial support from PUTI Pascasarjana (Contract Number: NKB-37/UN2.RST/HKP.05.00/2024) to cover research funding needs, and Lipoid-Germany for the phosphatidylcholine (Phospholipon 80G).

■ CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

■ AUTHOR CONTRIBUTIONS

Pedro Anugerah Aswan: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Data Curation. Baitha Palanggatan Maggadani: Validation, Investigation, Resources, Data Curation, Writing – Review & Editing. Wilzar Fachri: Software, Validation, Formal analysis, Visualization, Writing – Review & Editing. Raditya Iswandana: Conceptualization, Supervision, Project administration, Writing – Review & Editing, Funding acquisition. All authors agreed to the final version of the manuscript.

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