How to Cite: Dondulkar, A.O., Muley, M., Purohit, R.A., Yenorkar, N.Y., Akojwar, N.S., & Prasad, S.K. (2026). Simultaneous RP-HPLC Quantification and Characterization of Apigenin-Naringenin Co-Loaded ZnO Nanoparticles for Topical Delivery. *Eurasian Journal of Chemistry, 31, 2(122), 91–101.* https://doi.org/10.31489/2959-0663/2-26-3

Article

Received: 27 February 2026 | Revised: 28 April 2026 | Accepted: 29 April 2026 | Published online: 30 April 2026

UDC 546.47-31 : 543.544.5.068.7

https://doi.org/10.31489/2959-0663/2-26-3

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Simultaneous RP-HPLC Quantification and Characterization of Apigenin-Naringenin Co-Loaded ZnO Nanoparticles for Topical Delivery

Apigenin and Naringenin has proven beneficial prospects owing to an anti-inflammatory, antioxidant and wound healing effects. But having poor water solubility and low bioavailability hinders their application. Therefore, we developed a dual drug-loaded zinc oxide nanoparticle and validated a sensitive and selective liquid chromatography method for simultaneous determination. The nanoparticles were synthesized through chemical precipitation method followed by drug loading. They were evaluated for particle size, surface potential and FTIR, while morphology was determined by scanning electron microscopy. RP-HPLC method utilizing a C18 column as stationary phase and methanol/0.1 % orthophosphoric acid (75:25 v/v) as eluent at a flow rate of 1.0 mL/min with detection at 272 nm. Method was validated as per to ICH Q2(R1) guidelines and having linearity (5–25 µg/mL), accuracy, precision, specificity and robustness. The entrapment efficiencies of Apigenin and Naringenin were 88.6 ± 2.1 % and 85.2 ±1.8 %, respectively. The average size by dynamic-light scattering was 202.67 ± 3.2 nm and zeta potential were –30.51 ± 0.6 mV. Electron-microscopy confirmed mixture of mildly aggregated spherical and rod-shaped particles, size less than 200 nm. The validated analytical method and the nanoparticle system would together serve as a propitious groundwork, for combinatorial delivery of Apigenin and Naringenin.

Keywords: HPLC, polyphenols, simultaneous method, nanoparticles, apigenin, naringenin, isocratic, validation

1 Introduction

Polyphenols have garnered increasing attention in the treatment of various human diseases due to their safety profiles, wide accessibility, and cost-effectiveness [1]. Among them, natural flavonoids Apigenin and Naringenin have been shown to have potential therapeutic efficacy in the treatment of various pathological conditions, including diabetic wounds. Apigenin (4′,5,7-trihydroxyflavone), widely found in parsley, chamomile and celery, has anti-inflammatory, antioxidant, anti-diabetic, wound-healing potential. Another example is Naringenin (4′,5,7-trihydroxyflavanone), predominantly found in citrus fruits displays anti-inflammatory, antioxidant, and anti-microbial properties; as well as an inhibitory action on enzymes involved in releasing pro-inflammatory cytokines contributing to enhanced wound repair and tissue regeneration [2].

Apigenin and Naringenin have complementary pharmacological activities thereby making them suitable candidate to be co-delivered together in complex conditions as seen in diabetic wounds characterised by chronic inflammation, oxidative stress and impaired healing. However, water-insoluble, poorly available and rapidly metabolized limiting both compounds their therapeutic efficacies in clinical practice [3]. To overcome these challenges and harness their synergistic effects, we have established zinc oxide nanoparticles (ZnO NP) co-loaded formulation which not only provides stability for the drugs but also promotes controlled release mechanism and targets wound repair mechanisms under diabetic conditions. ZnO NPs are well established for their intrinsic antibacterial, anti-inflammatory, and skin healing properties that make them an ideal carrier system. Our formulation consists of adsorption of Apigenin and Naringenin over ZnO NPs resulting in a nano-sized system potentiated for better penetration and action at the wound site [4]. Currently, individual analytical methods are available for the quantification of Apigenin and Naringenin but a validated method is not reported simultaneously estimating them from nanoparticles-based delivery system. The pharmaceutical applications of dual-drug nanocarrier systems are increasing, especially for the management of complex disorders such as diabetic wounds; hence an established high throughput and non-destructive approach to support formulation development becomes imperative [5]. Simultaneous RP-HPLC methods are mainly

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essential because it improves the analytical throughput that leads to reducing simplified analysis time and reduced use of solvent compared to single drug estimation. It also enables precise examination of drug encapsulation, release kinetics and stability within the nanoparticle, which are vital for verifying therapeutic efficacy and batch-to-batch consistency. Furthermore, this methodology supports regulatory compliance by generating confirmed routine data for quality control purposes [6].

Considering these factors, our study focused on the development and validation of a robust, rapid, and accurate RP-HPLC method for the simultaneous quantification of Apigenin and Naringenin from co-loaded ZnO nanoparticles. The technique utilizes the isosbestic point, the wavelength at which both compounds show equal and maximum absorbance, to guarantee precise and consistent detection.

2 Experimental

2.1 Materials

Apigenin and Naringenin (purity >98 %) were purchased from Ottokemi and sigma Aldrich respectively, and used without further purification. Zinc precursor, such as zinc acetate dihydrate, was obtained from SRL chemicals. Methanol, acetonitrile, and water used for chromatographic analysis were of HPLC grade and sourced from merc. All other chemicals and reagents were of analytical grade. Dialysis membranes and 0.45 µm syringe filters were used during the nanoparticle purification process. All glassware was appropriately cleaned and dried before use.

2.2 Preparation and Characterization of Dual Drug-Loaded ZnO Nanoparticles

Zinc oxide (ZnO) nanoparticles were synthesized from zinc acetate using a simple wet chemical precipitation method. In this approach, zinc acetate dihydrate is initially dissolved in distilled water to form a clear solution, typically with a concentration between 0.1 M and 0.5 M. A separate solution of sodium hydroxide (NaOH) is then prepared, often with a slightly higher molarity (e.g., 0.2 M to 1 M), and added dropwise to the zinc acetate solution under continuous stirring. This leads to a white precipitate of zinc hydroxide ($Zn(OH)_2$), that gradually transforms into ZnO nanoparticles with progression in the reaction. Precipitation is done at pH 10–12 to have maximum precipitation. The reaction mixture had been stirred for a few hours at room temperature or mild heating (60–80 °C) to ensure complete conversion of $Zn(OH)_2$ to ZnO. Finally, the product is separated by centrifuge or filtration after aging, and then repeatedly washed with doubly distilled water and ethanol to remove impurities and then dried. The crystallinity was improved further by calcining the powder that has previously been dried using a temperature of 300–500 °C for 4 hours. This method is preferred due to its simplicity, low-cost and tunability of the nanoparticle size and morphology by varying concentration, pH, and temperature [7].

Apigenin and Naringenin were co-loaded on the nanoparticles. Each drug solution (1 mg/ml) was dissolved in ethanol and 5 mg of ZnO nanoparticles were taken in distilled water. Following that, the nanoparticle suspension was stirred for 800 rpm for 12 h; centrifuged under a speed of 8000 RPM for 20 min; then washed with distilled water to remove unbound drugs and reagents before dialyzed to eliminate other impurities. The pure formulation obtained after drying the suspension at 50 °C overnight, is stored in air tight containers for future use.

2.3 Characterization of Drug-Loaded ZnO Nanoparticles

The physicochemical characterization of the synthesized dual drug loaded ZnO nanoparticles is very important for stability, drug loading and biological performance. Attenuated Total reflectance (ATR) Infrared Spectroscopy (FTIR) with Shimadzu, was carried out in order to study any probable interaction between Apigenin, Naringenin, and ZnO nanoparticles. The FTIR spectra of ZnO nanoparticles loaded with the drugs were measured within the wavelength range of 4000–400 cm^{-1} through ATR-FTIR spectrophotometry, and the resulting peaks were correlated with their respective characteristic peaks from the literature. The particle size, polydispersity index (PDI), and zeta potential of these complexes were measured via Horiba scientific nanoparticle analyzer (SZ–100V2). The size distribution and homogeneity of the ZnO nanoparticles provide further insights into their proposed drug delivery mechanism. The surface morphology of the nanoparticles was observed by scanning electron microscopy (SEM). RP-HPLC method was used for the estimation of Apigenin and Naringenin entrapment efficiency in ZnO nanoparticles. This was done by centrifugation of the nanoparticle suspension and measuring the amount of free (unentrapped) drug in solution [8, 9]. All measurements were performed in triplicate ($n = 3$) using the same nanoparticle batch to evaluate analytical repeat-

ability. Batch-to-batch reproducibility of independent nanoparticle syntheses was not investigated in this study. Entrapment efficiency was calculated as follows:

$$\text{Entrapment Efficiency (\%)} = [(\text{Total drug} - \text{Free drug}) / \text{Total drug}] \times 100,$$

where Total drug is the initial amount of drug used for loading, Free drug is the amount of unentrapped drug present in the supernatant.

2.4 Instrumentation and Chromatographic Conditions

The RP-HPLC system employed for the chromatographic separation was Agilent 1100 (Agilent Technologies, Waldbronn, Germany) interfaced with Chemstation 10.01 software and a UV–Vis detector. We used Thermo C18 column with a dimension of 4.6 mm × 250 mm, particle size of 5.0 µm, at room temperature for separation of Apigenin and Naringenin. The mobile phase was a 75:25 mixture of methanol and 0.1 % OPA which was filtered and degassed before use. Flow rate was maintained at 1.0 mL/min, injection volume; 20 µL, detection at wave length of absorption of Apigenin and Naringenin (272 nm) which possess maximal absorbance and overlapping each other (isosbestic point). The full run time per sample was close. The column temperature was kept at 25 °C with a sample size of 20 µL.

2.5 Preparation of Standard and Sample Solutions

Stock standard solutions were prepared by dissolving 5 mg of each API in 10 mL of methanol giving STOCK-I with a final concentration of 500 µg/mL for both Apigenin and Naringenin. To validate the methodology, calibration range was set by serial dilution using working standard solutions. Quantities of drug-loaded ZnO nanoparticles were sonicated in methanol to achieve sample extraction for analysis. The resulting solution was filtered with 0.45 µm syringe filter and then injected in the system.

2.6 Method Validation

The developed RP-HPLC method used for simultaneous estimation of Apigenin and Naringenin from dual drug-loaded ZnO nanoparticles was validated as per International Council for Harmonisation (ICH Q2 (R1)) guidelines for analytical method validation. The method was validated as per ICH guidelines for linearity, accuracy, precision, LOD and LOQ, specificity, robustness and system suitability to ensure consistent analytical performance of the method.

The linearity was performed in the concentration range of 5–25 µg /mL by preparing the standard solutions of Apigenin and Naringenin at five different concentrations. The specified concentration range was selected considering the concentrations of Apigenin and Naringenin after the extraction process using nanoparticles, and was proven suitable for the best signal response, linearity, and sensitivity. Concentration levels: each concentration level was injected in triplicate, and calibration curves were constructed by plotting peak area against drug concentration. Linearity along with coefficients of Correlation (R^2) for both the drugs were carried out in the tested range.

The accuracy of the method was confirmed by recovery studies with three different levels: 80 %, 100 %, and 120 % of the target concentration. The quantities of Apigenin and Naringenin standards were spiked with the blank ZnO nanoparticle matrix, and their percent recovery was calculated by comparing the measured amounts to the quantity actually added. From there the mean recovery values of both drugs were verified.

The precision of the method was tested at two different levels namely intra-day (repeatability) and inter-day (intermediate precision). To determine the intra-day accuracy, same three concentrations (low, medium and high) were analysed $n = 3$ time in a day with similar condition. To evaluate inter-day precision, each sample was analysed on three different days by a single analyst on the same instrument to measure both within and between day variation.

Sensitivity for each drug was determined by calculating the Limit of Detection (LOD) and Limit of Quantification (LOQ) based on standard deviation of response (σ), and slope (S) of calibration curve utilizing the following equations:

$$\text{LOD} = 3.3\ (\times\sigma/S) \text{ and } \text{LOQ} = 10\ (\times\sigma/S).$$

The specificity was verified with the system suitability and also by analyzing blank nanoparticles of ZnO (drug-free) to see any interfering peak at Apigenin and Naringenin retention times. The method should be able to separate the analytes from other formulation excipients and nanoparticle components which will validate its specificity for simultaneous quantification of drugs.

Robustness was evaluated by making deliberate minor variations in chromatographic conditions, including flow rate ±0.1 mL/min, mobile phase composition ±2 %, and detection wavelength (±2 nm). These

variations did not significantly affect the retention times, peak areas, or resolution of the analytes, indicating the method's robustness and reliability under small operational deviations [10].

The ruggedness of the developed RP-HPLC method was assessed to determine its reproducibility under variable conditions such as different analysts and instruments. The study was conducted by performing replicate analyses of Apigenin and Naringenin standard solutions (15 µg/mL) on two different days, by two different analysts using two separate instruments of the same model [11].

Specificity was evaluated by analyzing blank ZnO nanoparticles (without drug) to confirm the absence of interference at the retention times of Apigenin and Naringenin. System suitability parameters were assessed before analysis to ensure consistent chromatographic performance. Parameters such as retention time, theoretical plate count, peak symmetry (tailing factor), and resolution between Apigenin and Naringenin peaks were evaluated. The results met the acceptance criteria, with resolution >2.0, theoretical plates >2000, and tailing factors <1.5, confirming the suitability of the system for routine analysis [12].

2.7 Data Analysis

The data were analysed statistically using Microsoft Excel (USA) to calculate the least squares (R^2), standard deviation, mean, relative standard deviation, and the equation of the calibration curve.

3 Results and Discussion

3.1 Optimization of Chromatographic Parameters

Various chromatographic parameters, such as the flow rate of mobile phase, the pH of the mobile phase, temperature and ratio between components of mobile phase were optimized by extensive analysis. After several trials, the optimized conditions were found as follows:

- flow rate of the mobile phase: 1.0 ml/min;
- column temperature: 25 °C;
- ratio of methanol and 0.1 % OPA (75:25);
- sample size: 20 µl;
- maximum pressure: 400 BAR;
- discharge rate: 0.001 to 5 ml.

Initially, different mobile phase compositions at various ratios resulted in poor resolution and the peak symmetry. For the simultaneous estimation of Apigenin and Naringenin, the isosbestic wavelength was set to 272 nm as shown in Figure 1 ensuring reliable and consistent quantification.

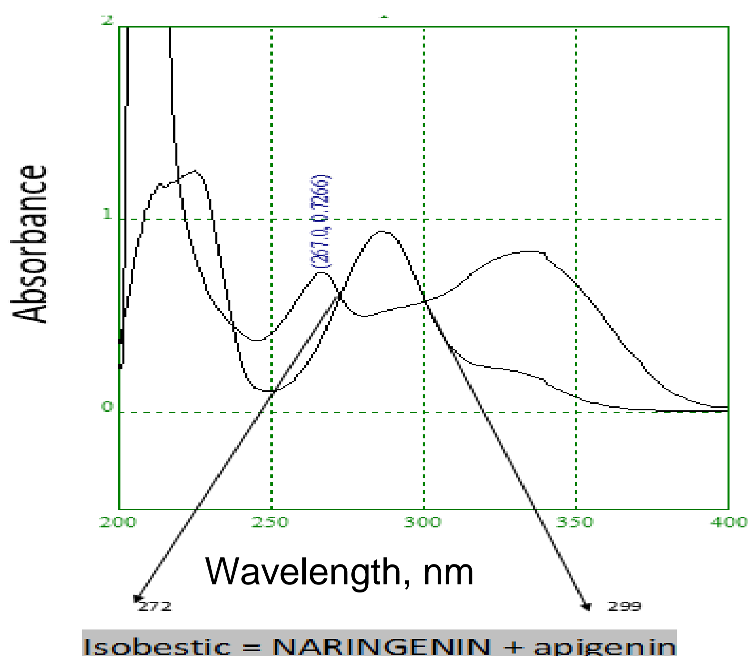

Figure 1. Absorption maxima showing isobestic point

The optimal retention times for Naringenin and Apigenin were 6.152 min and 7.539 min, respectively, with resolution of 4.24 as shown in Figure 2. The method exhibited good theoretical plate numbers and acceptable tailing factors, confirming efficient operation of the system. The chosen optimal parameters are applicable for the analysis of flavonoids [13].

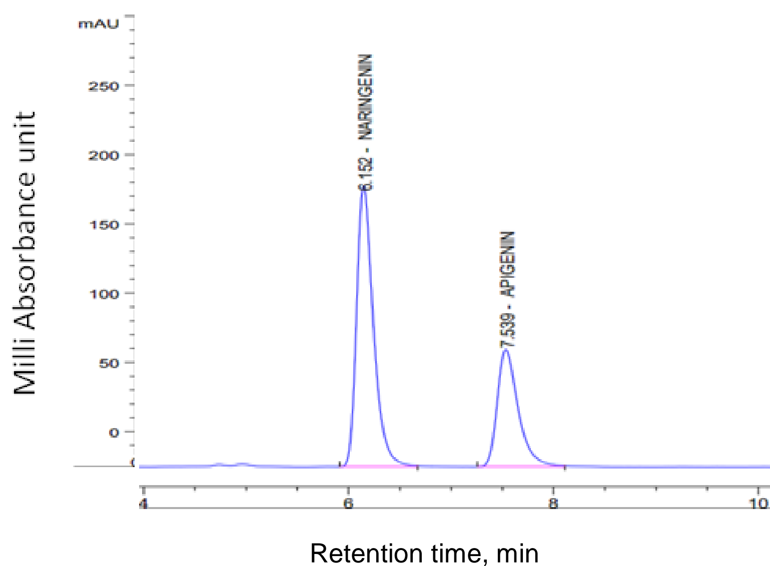

Figure 2. HPLC chromatogram for simultaneous estimation of Apigenin and Naringenin

3.2 Method Validation

For RP-HPLC method validation, the ICH guideline Q2(R1) was followed.

3.2.1 System Suitability Test

The mixture of Apigenin and Naringenin solution was injected into the HPLC column six times to assess the system suitability of the developed method. The analyzing concentration of both drugs in the mixture was 5μg/ml each. The average resolution of both drugs was 4.24, which indicates complete separation of the peaks. The RSD value for all parameters was less than 2 %, fulfilling both ICH and USP guidelines, confirming that the developed method is highly suitable and effective.

3.2.2 Linearity, Range, and Sensitivity

Five different concentrations of Apigenin (5–25 μg/ml) and Naringenin (5–25 μg/ml) were analysed separately. Each standard solution of six different concentrations was injected in three replicates and chromatographed. The AUCs of the different concentrations of both compounds were plotted against their respective concentrations in MS-Excel as shown in Figures 2 and 3. The calibration curve equations were as follows:

$$\text{Apigenin: } y = 83.811x - 26.353 \; (R^2 = 0.9984);$$

$$\text{Naringenin: } Y = y = 165.75x + 50.715 \; (R^2 = 0.9999).$$

The linear calibration curves showed excellent R^2 correlation coefficients (Apigenin $R^2 = 0.9984$ and Naringenin $R^2 = 0.9999$), indicating a direct linear proportionality between the concentration and the peak area. The limits of detection (LOD) and quantification (LOQ) for both drugs were calculated and found to be LOD for Apigenin 0.36 μg/ml and LOQ for Apigenin 1.0929 μg/ml, whereas LOD for Naringenin 0.2363 μg/ml and LOQ for Naringenin 0.7163 μg/ml. The values of LOD and LOQ obtained show that the method has adequate sensitivity for quantification purposes. The selected concentration range was adequate for practical purposes and falls within the expected range in the formulation of nanoparticles [14].

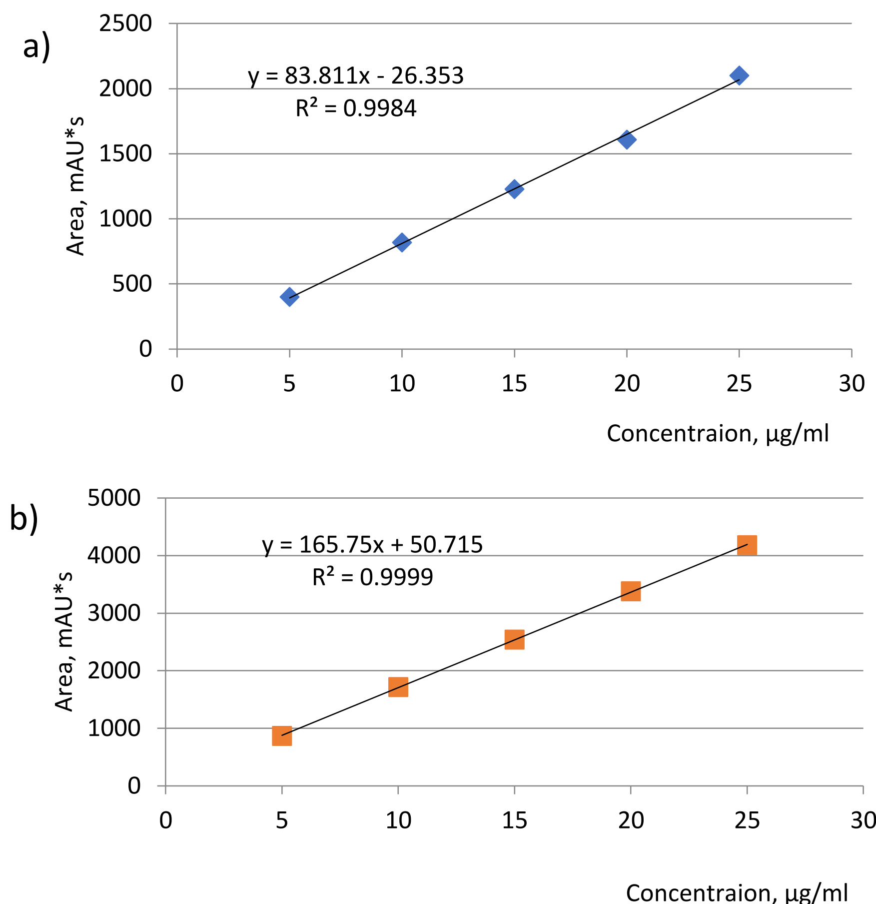

Figure 3. Linearity curve for *a*) Apigenin and *b*) Naringenin

3.2.3 Accuracy (Recovery Studies)

The accuracy of the developed RP-HPLC method was assessed through standard recovery experiments at three concentration levels: 80 %, 100 %, and 120 % of the target concentration. Known amounts of Apigenin and Naringenin were spiked into a blank ZnO nanoparticle matrix, and the recovered amounts were determined using the validated method. The recovery of Apigenin and Naringenin was found to be in between 98 % to 102 % which proved the reliability of the proposed method. The results are acceptable according to ICH standards and therefore can be used for the determination of analytes in the presence of matrix elements as presented in Table 1.

Table 1

Recovery study results of Apigenin and Naringenin values are mean of three replicates (*n* = 3)

Spike Level, %	Drug	Amount Added, µg/mL	Amount Recovered, µg/mL	Recovery, %	RSD, %
80 %	Apigenin	4.0	3.97	99.42	0.18
	Naringenin	4.0	3.96	99.03	0.36
100 %	Apigenin	5.0	4.99	99.36	0.63
	Naringenin	5.0	5.01	100.25	0.39
120 %	Apigenin	6.0	5.96	101.58	0.58
	Naringenin	6.0	6.03	101.58	0.05

3.2.4 Precision

Method precision was evaluated in terms of intra-day (repeatability) and inter-day (intermediate precision) by analyzing three different concentration levels (low, medium, and high) of both analytes (e.g., 5, 15, and 25 µg/mL). Intra-day precision was assessed by analyzing three replicates at each concentration within the same day, while inter-day precision was determined by repeating the procedure on three consecutive days

under identical conditions. The %RSD values for both intra-day and inter-day precision studies were below 2 %, indicating high reproducibility of the method. The low variability confirms that the method provides consistent results under the same and varying analytical conditions, fulfilling standard validation requirements, summarized in Table 2.

Table 2

Intraday and interday precision results for Apigenin and Naringenin values are mean of three replicates (*n* = 3)

Drug	Concentration, µg/mL	Intra-Day RSD, %	Inter-Day RSD, %
Apigenin	5	0.36	0.33
	15	0.13	0.07
	25	0.17	0.12
Naringenin	5	0.05	0.11
	15	0.15	1.11
	25	0.07	0.01

3.2.5 Robustness

The robustness of the implemented RP-HPLC technique was assessed by introducing little changes in essential chromatographic parameters systematically to ascertain that the process is still reliable, for regular quality control testing. Modifications were limited to minor adjustments of the flow rate (±0.1 mL/min), the mobile phase composition (±2 % methanol from optimal) and the detection wavelength (±2 nm from the most sensitive 272 nm). The variations were tested on a standard solution of Apigenin and Naringenin at the mid-level concentration (15 µg/mL). The newly developed technique was completely insensitive to small intentional changes in the experimental conditions like flow rate, mobile phase composition, and wavelength of detection. There was no noticeable change in any of the above-mentioned parameters, which indicated that the technique was quite robust [15]. Table 3 summarizes the results of the robustness study.

Table 3

Values shown are for 15 µg/mL of each drug. Resolution is reported only for Apigenin-Naringenin pair. values are mean of three replicates (*n* = 3)

<table>
<tr><th>Parameter</th><th>Conditions</th><th>Drug</th><th>Peak Area</th><th>Resolution</th><th>Plate Count</th><th>RSD, %</th></tr>
<tr><td rowspan="4">Flow rate</td><td rowspan="2">0.9 mL/min</td><td>Apigenin</td><td>1252.92</td><td rowspan="2">6.05</td><td>20703</td><td>0.26</td></tr>
<tr><td>Naringenin</td><td>2563.95</td><td>23563</td><td>0.11</td></tr>
<tr><td rowspan="2">1.1 mL/min</td><td>Apigenin</td><td>999.15</td><td rowspan="2">5.07</td><td>20043</td><td>0.05</td></tr>
<tr><td>Naringenin</td><td>2062.21</td><td>22785</td><td>0.17</td></tr>
<tr><td rowspan="4">Mobile phase ratio</td><td rowspan="2">74:26
(Methanol:0.1 % OPA)</td><td>Apigenin</td><td>1092.0</td><td rowspan="2">5.88</td><td>21360</td><td>0.04</td></tr>
<tr><td>Naringenin</td><td>2307.5</td><td>17899</td><td>0.05</td></tr>
<tr><td rowspan="2">76:24
(Methanol:0.1 % OPA)</td><td>Apigenin</td><td>1112.28</td><td rowspan="2">5.33</td><td>23523</td><td>0.12</td></tr>
<tr><td>Naringenin</td><td>2297.18</td><td>20712</td><td>0.04</td></tr>
<tr><td rowspan="4">Detection wavelength</td><td rowspan="2">271 nm</td><td>Apigenin</td><td>1037.8</td><td rowspan="2">5.60</td><td>24048</td><td>0.34</td></tr>
<tr><td>Naringenin</td><td>2229.6</td><td>21232</td><td>0.06</td></tr>
<tr><td rowspan="2">273 nm</td><td>Apigenin</td><td>1113.30</td><td rowspan="2">5.52</td><td>24024</td><td>0.30</td></tr>
<tr><td>Naringenin</td><td>2156.44</td><td>21375</td><td>0.10</td></tr>
</table>

3.2.6 Ruggedness

Peak area, retention time (*Rt*), resolution (*R*) and percentage relative standard deviation (*RSD*, %) of results were evaluated. The *RSD* values under all the conditions are less than 2 % which indicates that the method is rugged and also able to give reproducible results when a different laboratory person work on these matrices. These results verify the robustness of the approach for routine implementation.

3.2.7 Specificity

Contrary to formulation, the chromatograms had no co-eluting peaks or interfering signals near the corresponding retention times attesting that presence of excipients and nanoparticle matrix did not affect drug detection. No interference peaks were observed at the retention times of Apigenin and Naringenin, indicating

that the method was specific. The possibility of quantification of both substances in the presence of nanoparticle constituents indicates that the method is suitable for complex formulations.

3.2.8 Characterization of Drug-Loaded ZnO Nanoparticles

The physicochemical properties of the dual drug-loaded ZnO nanoparticles illustrated the successful formulation and stability. FTIR spectra of the drug-loaded ZnO nanoparticles shown in Figure 4*a*, revealed some distinctive peaks associated with both ZnO and drug-loaded particles. First, the broad peak observed near 3400 cm^{-1} is associated with O–H stretching modes, which shows that there are some phenolic groups (apigenin and naringenin) and hydroxyl groups on the surface of ZnO nanoparticles. Second, peaks near 1600–1650 cm^{-1} are related to aromatic C=C or C=O stretching modes of flavonoid compound structure. Finally, the peak near 500–600 cm^{-1} corresponds to Zn-O stretching. Compared with reported spectra of the individual components, slight shifts and changes in intensity were observed, suggesting successful drug loading through physical adsorption or weak intermolecular interactions rather than formation of new covalent bonds.

Upon drying, the nanoparticles were found to form a tan-coloured fine powder and were consistently able to disperse in an aqueous medium after mild sonication. The DLS results as depicted in Figure 4*b*, indicated that the average hydrodynamic diameter of the ZnO nanoparticles was 202.67 ± 3.2 nm, while the polydispersity index was 0.301, implying that the particle sizes were relatively close, thus suitable for topical applications. In addition, the zeta potential value of –30.51 ± 0.6 mV suggests high colloidal stability, thanks to electrostatic repulsions (Fig. 4*c*). Based on the SEM images (Fig. 5), the ZnO nanoparticles appeared spherical and rod-shaped, with some particles agglomerated, which is common among ZnO nanoparticles due to their high surface energy.

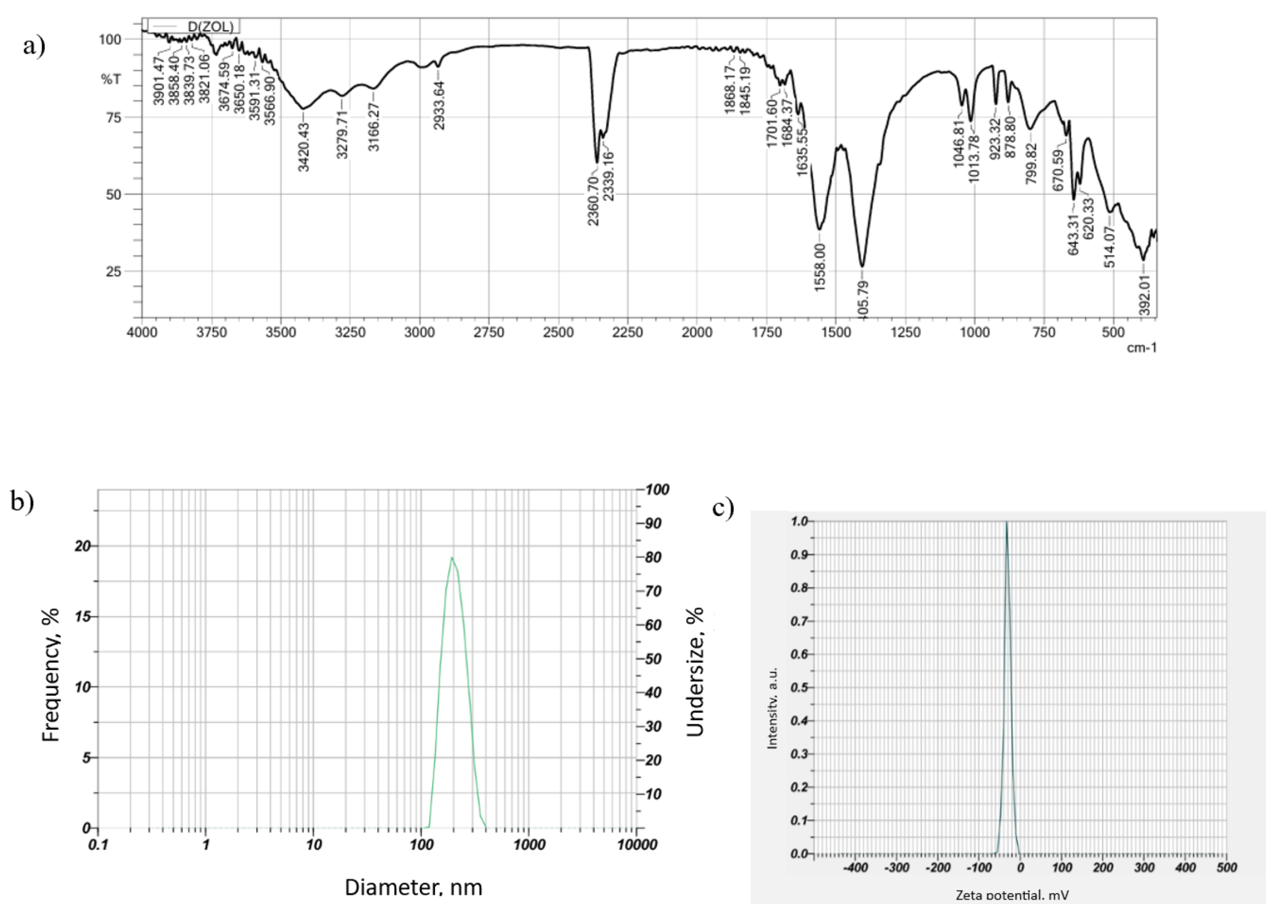

Figure 4. *a* — FTIR spectra of the apigenin-naringenin coloaded ZnO nanoparticles;
b — particle size distribution and *c* — zeta potential distribution of the apigenin-naringenin coloaded ZnO nanoparticles

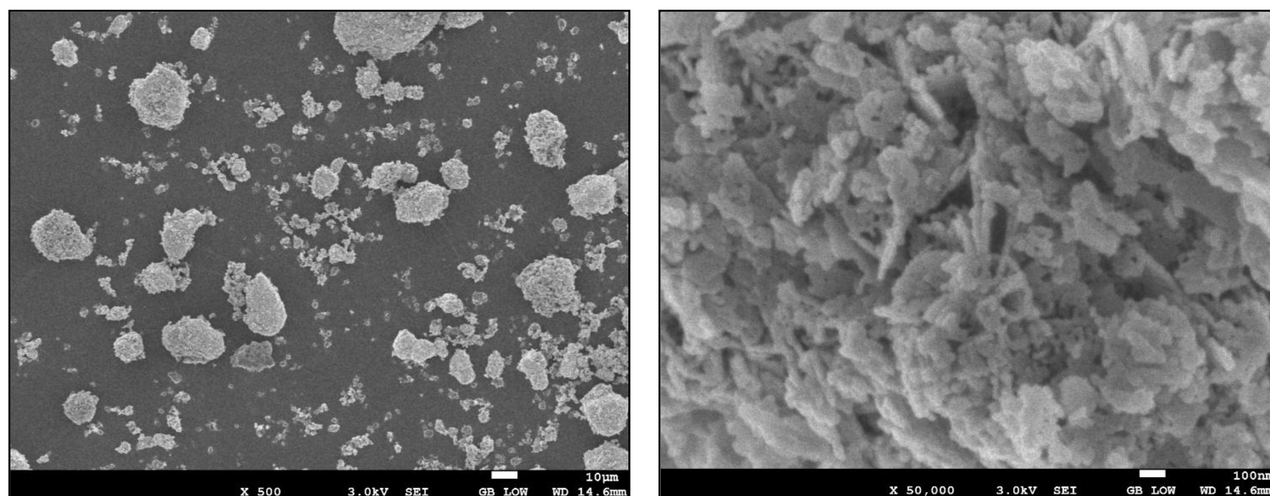

Figure 5. Scanning electron micrograms showing the morphology
of Apigenin-naringenin coloaded ZnO nanoparticles

3.2.9 Drug Entrapment Efficiency

Validated RP-HPLC method confirmed the entrapment efficiency of Apigenin and Naringenin in the ZnO nanoparticles. It was found that this method allows for the distinction of both drugs without interference from components present on the nanoparticle (Fig. 6). Apigenin and Naringenin entrapment efficiency results with both phytochemicals showing a high level of entrapment; apigenin had an average of 88.6 ± 2.1 % entrapment efficiency, naringenin also showed an 85.2 ± 1.8 %. These numbers reveal an efficient encapsulation of the drugs within the ZnO nanoparticle matrix and a probably high drug-ZnO surface interaction [7].

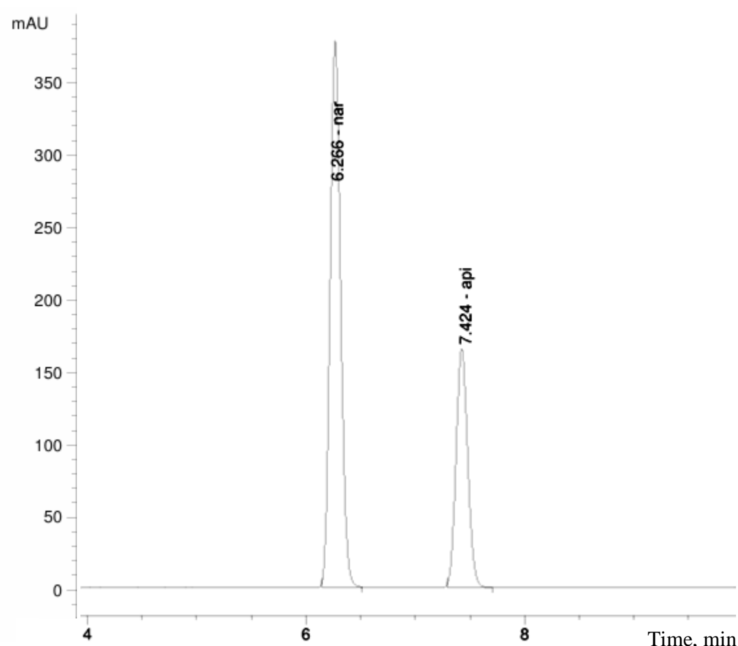

Figure 6. HPLC chromatogram for the drug entrapment study
of Apigenin-naringenin coloaded ZnO nanoparticles

4 Conclusions

The synthesized nanocrystalline ZnO NPs had nanosize dimension, good colloidal stability, and spherical shaped morphology. The high entrapment efficiency of Apigenin and Naringenin shows good encapsulation within the ZnO host matrix. A simple, rapid High-Performance Liquid Chromatography with Reversed Phase (RP-HPLC) method was successfully developed and validated for simultaneous estimation of

Apigenin and Naringenin from co-loaded ZnO nanoparticles. This method had a linear concentration dependence, was specific, sensitive and repeatable, and therefore could be used in routine quality analysis or *in vitro* studies.

In conclusion, the ZnO nanoparticle formulation was successful in loading two drugs and when used with a quantitative analytical method described here. It may be further optimized for sufficient delivery polyphenolic compounds to perform a sustained effect making it suitable for therapeutic applications including wound healing.

Funding

The authors wish to sincerely acknowledge the financial assistance provided by DST-PURSE, Government of India. This work is supported by PAIR network of SAKSHAM funded by ANRF (Anusandhan National Research Foundation), India, through grant ANRF/PAIR/ 2025/000018/PAIR, administered and mentored by IIT Indore.

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Authors Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Ayusha Omprakash Dondulkar** conceptualization, data creation, investigation, methodology, validation writing original draft; **Mandar Mahendra Muley** data creation, **Raksha Anand Purohit** Writing review and editing; **Nikhil Yadavrao Yenorkar** Visualization and editing; **Natasha Sudhir Akojwar** Review and editing; **Satyendra Kuldeep Prasad** conceptualization, review, supervision and communication.

Acknowledgement

Authors would like to acknowledge the fellowship awarded by Mahatma Jyotiba Phuley Research Fellowship, Government of Maharashtra, India to Ms. Ayusha O. Dondulkar (Letter No.: Fellowship2022_973 dated 13/12/2022).

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability Statement

All data supporting the findings of this study are available within the article or its supplementary materials.

References

1 Ghasemi, J., Shamsabadi, S., Mahzoon, E., Nazer, Y., Rahimi, V.B., & Askari, V.R. (2024). A Mechanistic Insight into the Effects of Marine Sources as Functional Foods Against Metabolic Syndrome: A Review Article. *Letters in Drug Design & Discovery*, *21*(19), 4668–4682. https://doi.org/10.2174/0115701808344500250115114637

2 Li, J., Mao, B., Tang, X., Zhang, Q., Zhao, J., Zhang, H., & Cui, S. (2023). Protective effects of naringenin and apigenin in ameliorating skin damage via mediating the Nrf2 and NF-κB pathways in mice. *Foods*, *12*(11), 2120. https://doi.org/10.3390/foods12112120

3 Yoon, J.H., Kim, M.Y., & Cho, J.Y. (2023). Apigenin: A therapeutic agent for treatment of skin inflammatory diseases and cancer. *International Journal of Molecular Sciences*, *24*(2), 1498. https://doi.org/10.3390/ijms24021498

4 Xie, J., Li, H., Zhang, T., Song, B., Wang, X., & Gu, Z. (2023). Recent advances in ZnO nanomaterial-mediated biological applications and action mechanisms. *Nanomaterials*, *13*(9), 1500. https://doi.org/10.3390/nano13091500

5 Gouda, A., Sakr, O.S., Nasr, M., & Sammour, O.A. (2024). Developing a rapid analytical method for simultaneous determination of apigenin and gallic acid: validation and application in a nanoliposomal formulation. *Drug Development and Industrial Pharmacy*, *50*(3), 274–283. https://doi.org/10.1080/03639045.2024.2318386

6 Bhia, M., Motallebi, M., Abadi, B., Zarepour, A., Pereira-Silva, M., Saremnejad, F., ... & Shakibaei, M. (2021). Naringenin nano-delivery systems and their therapeutic applications. *Pharmaceutics*, *13*(2), 291. https://doi.org/10.3390/pharmaceutics13020291

7 Manyasree, D., Kiranmayi, P., & Venkata, R.K. (2018). Characterization and antibacterial activity of ZnO nanoparticles synthesized by co-precipitation method. *International Journal of Applied Pharmaceutics,* *10*(6), 224–228. http://dx.doi.org/10.22159/ijap.2018v10i6.29376

8 Mandal, A.K., Katuwal, S., Tettey, F., Gupta, A., Bhattarai, S., Jaisi, S., Bhandari, D.P., Shah, A.K., Bhattarai, N., & Parajuli, N. (2022). Current Research on Zinc Oxide Nanoparticles: Synthesis, Characterization, and Biomedical Applications. *Nanomaterials, 12*(17), 3066. https://doi.org/10.3390/nano12173066

9 Rajabi, H.R., Naghiha, R., Kheirizadeh, M., Sadatfaraji, H., Mirzaei, A., & Alvand, Z.M. (2017). Microwave assisted extraction as an efficient approach for biosynthesis of zinc oxide nanoparticles: synthesis, characterization, and biological properties. *Materials Science and Engineering: C*, *78*, 1109–1118. https://doi.org/10.1016/j.msec.2017.03.090

10 Chaudhari, V.S., Borkar, R.M., Murty, U.S., & Banerjee, S. (2020). Analytical method development and validation of reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous quantifications of quercetin and piperine in dual-drug loaded nanostructured lipid carriers. *Journal of Pharmaceutical and Biomedical Analysis*, *186*, 113325. https://doi.org/10.1016/j.jpba.2020.113325

11 Bhutia, G.T., Kumar DE.A., & Bera, T. (2022). Validation, stability studies, and simultaneous estimation of co-encapsulated curcumin, epigallocatechin gallate nanoformulation by RP-HPLC method. *International Journal of Applied Pharmaceutics*, *14*, 186–195. https://dx.doi.org/10.22159/ijap.2022v14i6.45818

12 Khismatrao, A., Bhairy, S., & Hirlekar, R. (2018). Development and validation of RP-HPLC method for simultaneous estimation of curcumin and piperine. *International Journal of Applied Pharmaceutics*, *10*(5), 43. https://doi.org/10.22159/ijap.2018v10i5.21140

13 Patil, C., Naik, P., Mallamma, T., & Goudanavar, P. (2025). Exploring the potential of a quick and simultaneous DoE-based stability indicating novel RP-HPLC method for the estimation of capecitabine and curcumin in biodegradable nanoparticles and human plasma. *Journal of Chromatography B*, 124731. https://doi.org/10.1016/j.jchromb.2025.124731

14 Khatak, S., Khatak, M., Ali, F., Rathi, A., Singh, R., Singh, G.N., & Dureja, H. (2018). Development and Validation of a RP-HPLC Method for Simultaneous Estimation of Antitubercular Drugs in Solid Lipid Nanoparticles. *Indian Journal of Pharmaceutical Sciences*, 80(6). https://doi.org/10.4172/pharmaceutical-sciences.1000449

15 Venishetty, V.K., Parikh, N., Sistla, R., Ahmed, F.J., & Diwan, P.V. (2011). Application of validated RP-HPLC method for simultaneous determination of docetaxel and ketoconazole in solid lipid nanoparticles. *Journal of Chromatographic Science*, *49*(2), 136–141. https://doi.org/10.1093/chrsci/49.2.136