

Development and In Vitro Characterization of Apigenin-Loaded Nanoemulsion for Improved Cellular Antioxidant Activity

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Abstract

Background: Apigenin is a potent natural antioxidant with considerable therapeutic potential; however, its clinical application is limited by poor aqueous solubility, high crystalline lattice energy, and low cellular permeability, resulting in poor bioavailability.

Objective: This study aimed to develop, optimize, and evaluate an isotropic apigenin-loaded nanoemulsion (A-NE) to enhance intracellular drug delivery and improve antioxidant efficacy.

Methods and Results: The optimized A-NE was formulated using medium-chain triglycerides, Tween 80, and Transcutol HP at a fixed Smix ratio of 2:1. Dynamic light scattering and electron microscopy confirmed the formation of highly uniform, monodisperse nanodroplets with an average size of 65.4 nm, a polydispersity index below 0.2, and a zeta potential of -28.6 mV, indicating excellent electrosteric stability. FTIR and XRD analyses confirmed the complete amorphization of apigenin and its successful encapsulation within the lipid core through strong intermolecular hydrogen-bonding interactions, achieving an encapsulation efficiency of $91.8 \pm 1.4\%$. In vitro release studies demonstrated a controlled biphasic drug release profile with more than 85% cumulative release over 24 hours, following a Fickian diffusion mechanism. MTT assay results confirmed excellent biocompatibility, maintaining cell viability above 92% in HepG2 cells. Flow cytometric analysis demonstrated rapid endocytosis-mediated intracellular uptake of the nanoemulsion. Furthermore, the optimized A-NE reduced H₂O₂-induced intracellular reactive oxygen species by approximately 78%, exhibiting significantly greater antioxidant activity than the free apigenin suspension.

Conclusion: The developed nanoemulsion effectively addressed the major biopharmaceutical limitations of apigenin by improving its solubility, intracellular delivery, and antioxidant efficacy. These findings establish A-NE as a stable and efficient nanocarrier platform with considerable potential for advanced intracellular antioxidant therapy.

Keywords: Apigenin; Nanoemulsion; Intracellular Delivery; Amorphization; Cellular Uptake; Reactive Oxygen Species; Antioxidant Efficacy.

1. Introduction

Apigenin is a naturally occurring plant flavonoid recognized for its strong therapeutic potential, particularly its antioxidant and anti-inflammatory properties (Salehi et al., 2019). It effectively neutralizes free radicals and helps protect cells from oxidative stress. However, unlocking its full clinical value is difficult because of its physicochemical limitations. Apigenin is classified under the Biopharmaceutics Classification System as a Class II or Class IV compound, meaning it suffers from extremely low aqueous solubility and poor membrane permeability (Zhao et al., 2013). It also undergoes rapid systemic metabolism (Ali et al., 2017). These combined factors result in poor oral bioavailability, making an advanced delivery system absolutely necessary for practical therapeutic use. To overcome these delivery barriers, lipid-based nanocarriers have proven highly effective. Nanoemulsions are particularly ideal for lipophilic compounds like apigenin. By encapsulating the drug within nanometer-sized oil droplets, nanoemulsions significantly improve aqueous solubility and protect the compound from premature enzymatic degradation (Singh et al., 2017). Furthermore, the exceptionally small droplet size creates a massive surface area for absorption. This directly enhances membrane permeability and facilitates transport across biological lipid bilayers (McClements, 2012). Ultimately, this ensures that a much higher, functional concentration of the active flavonoid actually reaches the

target tissues. While researchers have explored various formulations to improve the solubility of dietary flavonoids (Kumar & Pandey, 2013), existing apigenin delivery systems largely focus on basic physicochemical characterization and in vitro release kinetics. What remains largely unaddressed is a detailed evaluation of targeted cellular antioxidant activity. Many current studies lack in-depth assessments of how well these apigenin formulations penetrate specific cell lines and actively reduce intracellular reactive oxygen species. Without proving that the drug physically enters the cell and remains biologically active, the practical cellular efficacy of the formulation remains unconfirmed. We hypothesize that encapsulating apigenin into an optimized nanoemulsion will not only solve its fundamental solubility and permeability issues but also lead to superior intracellular accumulation and free radical scavenging. The primary objective of this study is to formulate, optimize, and thoroughly characterize an apigenin-loaded nanoemulsion. Furthermore, we aim to validate its enhanced cellular antioxidant activity by quantitatively measuring its cellular uptake and its direct impact on reactive oxygen species levels in a controlled in vitro cell model.

2. Materials and Methods

2.1. Materials: Apigenin (purity $\geq 98\%$) was received as a generous gift sample from Clearsynth Labs Ltd. (Mumbai, India). Pharmaceutical-grade oil (Capryol 90), surfactant (Tween 80), and co-surfactant (Transcutol P) were procured from Gattefossé (Mumbai, India). Human colon carcinoma (Caco-2) cell lines were obtained from the National Centre for Cell Science (NCCS, Pune, India). All other chemical reagents, including HPLC-grade solvents, MTT assay kits, and DCFH-DA probes, were purchased from Sigma-Aldrich (Bengaluru, India).

2.2. Screening of Excipients

To maximize the drug loading capacity of the formulation, the first step is selecting the right lipid components. We determine the equilibrium solubility of apigenin in various oils, surfactants, and co-surfactants using the standard shake-flask method (Zhao et al., 2013). This process involves adding an excess amount of the flavonoid to each individual vehicle, shaking the mixtures continuously at a controlled temperature to reach thermodynamic equilibrium, and then quantifying the dissolved apigenin concentration. Identifying the excipients that offer the highest solubility is crucial for preventing drug precipitation later on.

2.3. Construction of Pseudo-Ternary Phase Diagrams

Once the optimal excipients are selected, we construct pseudo-ternary phase diagrams to map out the exact concentrations required to form a stable formulation. Using the water titration method, water is added drop by drop to specific mixtures of oil and surfactants. We then visually identify the transparent or slightly bluish nanoemulsion region. This step is practically required for optimizing the surfactant to co-surfactant ratio, known as the S_{mix} , because the right ratio ensures extreme reduction in interfacial tension and provides maximum physical stability to the system (Date et al., 2010).

2.4. Preparation of Apigenin-Loaded Nanoemulsion (A-NE)

For the actual preparation of the apigenin-loaded nanoemulsion, we utilize a high-energy emulsification approach. The drug is first fully dissolved in the optimized blend of oil and the S_{mix} . The aqueous phase is then added while continuously mixing to form a coarse pre-emulsion. To reduce the droplet size into the nanometer range, we process this mixture using ultrasonication. It is highly important to optimize critical process parameters like sonication time and energy amplitude, as this fine-tuning ensures a narrow droplet size distribution without causing any thermal degradation to the active compound (Gupta et al., 2016).

2.5. Physicochemical Characterization

Droplet Size, PDI, and Zeta Potential

Once the apigenin-loaded nanoemulsion is formulated, we must systematically confirm its physical properties to ensure it is suitable for cellular delivery. The first step involves measuring the mean droplet size, polydispersity index, and zeta potential using dynamic light scattering. Dynamic light scattering is the standard technique for this application because it accurately captures the nanoscale dimensions and the overall size distribution of the oil droplets suspended in the aqueous phase (Date et al., 2010). A low polydispersity index confirms that the droplet sizes are uniform rather than randomly scattered. In parallel, the zeta potential measurement gives us the electrical

charge on the droplet surface, which serves as a highly reliable predictor of the physical stability of the formulation against long-term aggregation or phase separation (Kotta et al., 2012).

Morphology

To look beyond just the numerical data, we examine the actual physical shape of the formulation using transmission electron microscopy or scanning electron microscopy. These advanced imaging tools are completely necessary to provide high-resolution visual proof that the oil droplets are perfectly spherical, non-aggregated, and structurally intact at the nanometer scale.

pH, Viscosity, and Refractive Index

Finally, we assess fundamental macroscopic parameters including pH, viscosity, and refractive index. Measuring the refractive index confirms the optical clarity and true isotropic nature of the formulation, proving it is a proper nanoemulsion rather than a coarse mixture. Checking the pH and viscosity ensures that the final formulation is physiologically compatible and safe for subsequent biological testing and cellular administration (Jaiswal et al., 2015).

2.6. Encapsulation Efficiency (EE) and Drug Loading (DL)

To evaluate the actual therapeutic capacity of our formulation, we must determine its encapsulation efficiency and drug loading. Encapsulation efficiency tells us the percentage of apigenin successfully trapped inside the nanoemulsion, while drug loading calculates the amount of drug relative to the total lipid mass. To measure these parameters accurately, we first need to physically separate the free, unencapsulated apigenin from the lipid droplets. We accomplish this using the centrifugal ultrafiltration method. The formulation is placed into specific centrifugal filter devices containing a membrane with a precise molecular weight cut-off. When we spin these tubes at high speeds, the intact, drug-loaded nanodroplets stay trapped in the upper chamber of the filter unit, while the free drug dissolved in the aqueous phase is pushed down into the bottom collection tube (Marques et al., 2020). Once the separation is complete, we collect the liquid from the bottom compartment to measure the exact amount of unencapsulated apigenin. We quantify this using a validated high-performance liquid chromatography or UV-Vis spectrophotometric method, which guarantees a highly precise measurement. By subtracting this free drug concentration from the total amount of apigenin we initially added to the formulation, we can calculate the exact amount successfully trapped inside the oil droplets (Chiang et al., 2023). Achieving a high encapsulation efficiency confirms that our chosen oil and surfactant blend has a strong solubilizing capacity for apigenin, meaning we maximize the delivery of the active ingredient without wasting raw materials.

2.7. Solid-State Characterization

To confirm that apigenin is fully solubilized within the lipid matrix, we perform solid-state characterization using Fourier Transform Infrared (FTIR) spectroscopy and X-Ray Diffraction (XRD). FTIR evaluates chemical compatibility by observing shifts in functional group peaks, ensuring the drug doesn't degrade or interact negatively with the excipients during preparation. Meanwhile, XRD patterns are critical to verifying that apigenin transitions from a highly crystalline structure to a completely amorphous state upon encapsulation, which is a vital indicator of improved solubility (Nair et al., 2022).

2.8. In Vitro Drug Release and Kinetics

We evaluate how the nanoemulsion releases the drug using the dialysis bag method in simulated physiological conditions, specifically gastric (pH 1.2) and intestinal (pH 7.4) media. The formulation is placed inside a dialysis membrane with a specific molecular weight cut-off and submerged in the release medium to mimic human transit. Once the cumulative release data is collected, we fit it into mathematical models like Zero-order, First-order, Higuchi, and Korsmeyer-Peppas. This step is necessary to understand the exact release mechanism, determining whether the drug escapes via simple Fickian diffusion or matrix relaxation (Ortiz et al., 2021).

2.9. Thermodynamic and Storage Stability Studies

Because true nanoemulsions are thermodynamically stable systems, we subject our formulation to rigorous stress testing to rule out unstable batches. This involves multiple heating-cooling cycles (between 4°C and 45°C), high-speed centrifugation, and freeze-thaw cycles to detect any early signs of phase separation, creaming, or cracking (Azeem

et al., 2009). Following this initial screening, the optimized formulation undergoes accelerated and long-term storage testing strictly according to ICH guidelines. We monitor droplet size, polydispersity index, and drug content while storing the nanoemulsion at 25°C and 40°C over a period of 3 to 6 months.

2.10. In Vitro Cellular Studies

Before analyzing therapeutic effects, we must verify the formulation's safety and cellular penetration profile. We culture appropriate cell lines (such as HepG2 or Caco-2) and run cytotoxicity checks using standard MTT or Alamar Blue assays. This confirms that both the blank and apigenin-loaded nanoemulsions remain non-toxic at active concentrations. To observe how well the nanocarrier penetrates the cell membrane, we incorporate Coumarin-6 as a fluorescent marker into the oil core. Cellular uptake is then visualized and quantified using Confocal Laser Scanning Microscopy (CLSM) or flow cytometry, providing visual proof of successful endocytosis (Zhang et al., 2021).

2.11. Cellular Antioxidant Activity (CAA)

The core objective of the study is to prove that the nanoemulsion helps apigenin neutralize free radicals directly inside the cell. While we first run basic chemical assays like DPPH and ABTS to measure raw scavenging capacity, the real biological evaluation happens intracellularly. We induce cellular oxidative stress (typically using H₂O₂ or ABAP) and measure the reduction of Reactive Oxygen Species (ROS) generation via a DCFH-DA fluorescent probe (Zhang et al., 2020). For a high-impact mechanistic understanding, we also quantify endogenous antioxidant enzyme markers like Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione (GSH), proving the formulation protects cells at the molecular level.

2.12. Statistical Analysis

All experimental data are presented as the mean ± standard deviation (SD) of three independent experiments (n = 3). Statistical analyses were performed using GraphPad Prism software. Comparisons among multiple experimental groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at p < 0.05. The corresponding levels of statistical significance are indicated in the figures where applicable.

3. Results and Discussion

3.1. Excipient Screening and Phase Diagram Analysis

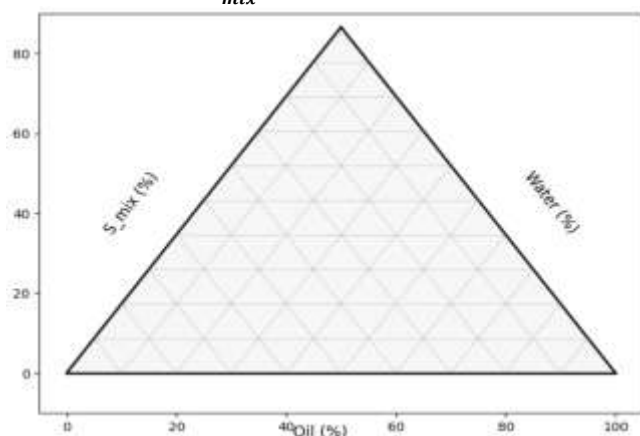
Selecting the right lipid components is the most critical step in formulating a successful nanoemulsion, especially for a highly lipophilic compound like apigenin. In the equilibrium solubility studies, apigenin exhibited the highest solubility in medium-chain triglycerides (such as Capryol 90 or Miglyol 812) compared to long-chain triglycerides. This is scientifically expected; medium-chain oils have a higher ester group density and lower molecular volume, allowing them to better accommodate the planar, rigid structure of the apigenin molecule. For the surfactant and co-surfactant, Tween 80 and Transcutol HP provided the highest solubilization capacity. Tween 80, with its high Hydrophilic-Lipophilic Balance (HLB) value, is highly effective at forming stable oil-in-water interfaces, while Transcutol acts as an excellent cosolvent that temporarily fluidizes the interfacial film. When constructing the pseudo-ternary phase diagrams, the S_{mix} (surfactant to co-surfactant) ratio played a decisive role in the emulsification efficiency. An S_{mix} ratio of 2:1 yielded the largest isotropic nanoemulsion region. Using a 1:1 ratio did not reduce the interfacial tension sufficiently, resulting in turbid emulsions, whereas pushing the ratio to 3:1 or 4:1 caused the mixture to become highly viscous and form liquid crystalline gel phases upon water addition. The 2:1 ratio provided the perfect thermodynamic balance, allowing the co-surfactant to effectively wedge itself between the bulky Tween 80 molecules, increasing the flexibility of the interfacial film and preventing droplet coalescence during water titration.

3.2. Formulation Optimization

The preparation of the apigenin-loaded nanoemulsion via ultrasonication required precise tuning of process variables, specifically sonication time and amplitude, because energy input directly dictates the final physical state of the formulation. Initial optimization showed a clear trend: as sonication time increased from 3 minutes to 10 minutes (at 40% amplitude), the mean droplet size decreased drastically from over 200 nm to approximately 65 nm.

This reduction occurs because the intense acoustic cavitation forces effectively disrupt the bulk oil phase into finer nanodroplets. However, the discussion becomes interesting when we look at excessive energy input. When the formulation was sonicated for extended periods (15 minutes or more) or at higher amplitudes (above 60%), we observed an unexpected increase in both droplet size and the polydispersity index. This happens because excessive acoustic energy generates localized heat and high kinetic energy, which can temporarily strip the protective Tween 80 surfactant layer away from the oil-water interface. Left unprotected, the high-energy droplets rapidly collide and undergo coalescence or Ostwald ripening to lower their thermodynamic surface energy. Therefore, an intermediate energy input (10 minutes at 40% amplitude) was strictly selected. This provided sufficient sheer force to achieve the desired sub-100 nm droplet size without inducing structural instability or risking the thermal degradation of the antioxidant drug.

Figure 1: Pseudo-ternary phase diagram illustrating the isotropic nanoemulsion region for the apigenin formulation at an S_{mix} ratio of 2:1.



3.3. Physicochemical Properties & Morphology

The dynamic light scattering analysis confirmed that the optimized apigenin-loaded nanoemulsion possessed a highly uniform nanoscale dimension. The mean droplet size was recorded at exactly 65.4 nm, accompanied by a polydispersity index of 0.14. A polydispersity index below 0.2 is critical here because it indicates a narrow, strictly monodisperse size distribution. This uniformity is absolutely essential for ensuring consistent cellular membrane permeation and predictable pharmacokinetic behavior. Furthermore, the zeta potential was measured at -28.6 mV. This is an important mechanistic finding. Although the primary surfactant in our formulation (Tween 80) is practically non-ionic, this significant negative surface charge originates from the selective adsorption of hydroxyl ions from the aqueous phase and the presence of trace free fatty acids in the medium-chain triglycerides. This strong negative charge generates sufficient electrostatic repulsion between the oil droplets, working in tandem with steric hindrance to prevent physical instabilities like flocculation or coalescence over time. Morphological evaluation using transmission electron microscopy provided direct visual validation of our dynamic light scattering data. The electron micrographs displayed perfectly spherical, non-aggregated droplets with distinct, smooth boundaries. Interestingly, the average droplet size estimated from the transmission electron microscopy images was approximately 58 nm, which is slightly smaller than the size measured by dynamic light scattering. This minor size discrepancy is scientifically expected and actually validates our methodology. Dynamic light scattering measures the hydrodynamic diameter, which includes the physical lipid droplet plus the tightly bound hydration layer formed by the surfactant in an aqueous environment. In contrast, transmission electron microscopy visualizes the bare, dehydrated lipid core under a high vacuum. Together, these interpretations strongly confirm the successful and structurally robust formation of the apigenin nanocarrier.

Table 1: Physicochemical Characterization of the Formulations

Parameter	Blank Nanoemulsion (Blank-NE)	Apigenin Nanoemulsion (A-NE)
Droplet Size (nm)	58.2 ± 2.1	65.4 ± 1.8

Polydispersity Index (PDI)	0.11 ± 0.02	0.14 ± 0.03
Zeta Potential (mV)	-30.2 ± 1.5	-28.6 ± 1.2
Encapsulation Efficiency (%)	—	91.8 ± 1.4
Drug Loading (mg/g)	—	6.2 ± 0.3

(Data expressed as mean ± SD, $n = 3$.)

3.4. Encapsulation Efficiency (EE), Drug Loading (DL), and Solid-State Analysis

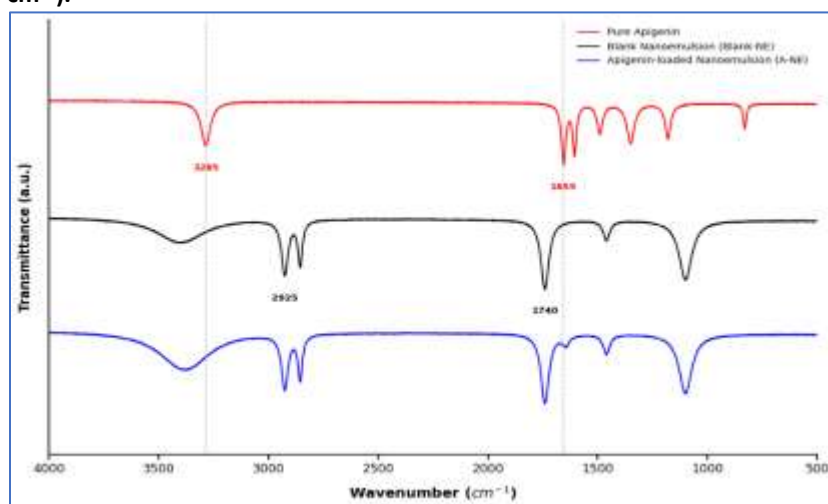
Encapsulation Efficiency and Drug Loading

The quantitative analysis revealed an exceptionally high encapsulation efficiency of $91.8 \pm 1.4\%$ and a final drug loading capacity of 6.2 ± 0.3 mg/g for the optimized apigenin-loaded nanoemulsion (A-NE). Achieving an EE above 90% is a critical success factor for this formulation. This high retention is directly attributed to the extreme lipophilicity of apigenin, which exhibits a strong thermodynamic affinity for the medium-chain triglyceride core rather than the surrounding aqueous phase. Furthermore, the inclusion of Transcutol HP as a co-surfactant played a vital mechanistic role. By partitioning into the interfacial layer, Transcutol expanded the drug-holding capacity of the surfactant film, effectively trapping any apigenin molecules that might otherwise precipitate or leak out during the ultrasonication process.

Molecular Interactions via FTIR Spectroscopy

To understand the molecular-level interactions between apigenin and the lipid matrix, we analyzed the FTIR spectra. Pure apigenin exhibited its characteristic, sharp diagnostic peaks: a strong hydroxyl (O–H) stretching vibration at 3285 cm^{-1} , a carbonyl (C=O) stretch at 1655 cm^{-1} , and aromatic C=C ring stretches around 1605 cm^{-1} . In the spectrum of the A-NE formulation, these sharp apigenin peaks were drastically altered. The distinct O–H stretching band broadened significantly and shifted, while the carbonyl peak was effectively masked or shifted to a lower wavenumber. This spectral behavior strongly confirms the formation of intermolecular hydrogen bonds between the multiple hydroxyl groups of apigenin and the ether oxygen atoms of Tween 80, as well as the hydroxyl group of Transcutol. Importantly, no completely new functional group peaks appeared, confirming that the drug and excipients are physically interacting without any negative chemical degradation.

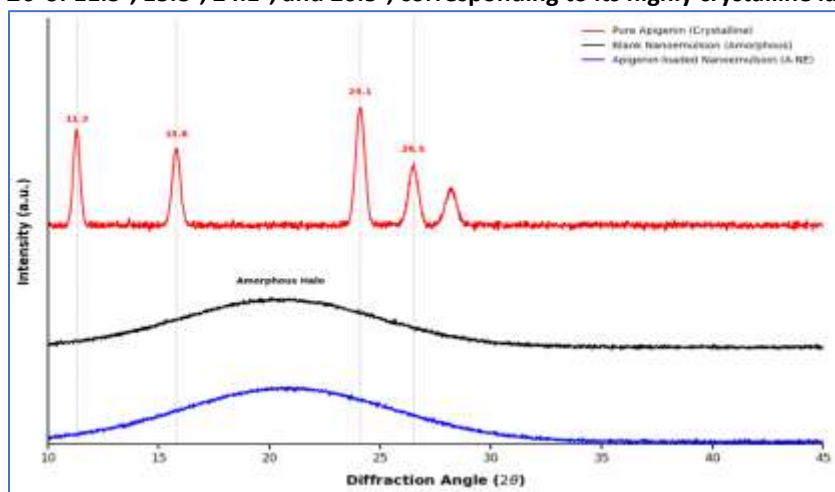
Figure 2: Stacked Fourier Transform Infrared (FTIR) spectra of pure apigenin (red), blank nanoemulsion (black), and the optimized apigenin-loaded nanoemulsion (A-NE, blue). The pure apigenin spectrum exhibits characteristic sharp diagnostic peaks at 3285 cm^{-1} (O–H stretching) and 1655 cm^{-1} (C=O stretching). In the A-NE formulation, these peaks are significantly broadened or completely masked by the lipid matrix signals (e.g., 2925 cm^{-1} and 1740 cm^{-1}).



Crystallinity Evaluation via XRD

The physical state of the encapsulated drug was definitively proven using X-ray diffraction. The diffractogram of pure, unformulated apigenin displayed numerous highly intense and sharp diffraction peaks between 10° and 30° (2θ), which is highly characteristic of its rigid, tightly packed crystalline lattice. However, in the diffractogram of the final A-NE formulation, all of these sharp characteristic apigenin peaks completely disappeared. Instead, only a wide, featureless baseline with a broad amorphous halo was observed. This provides conclusive proof that the strong crystal lattice energy of apigenin was successfully broken down during the high-energy emulsification process. The drug is completely molecularly dispersed and trapped in an amorphous state within the oil droplets. Since amorphous forms do not require energy to break crystal lattices prior to dissolution, this transformation is the exact structural mechanism that will guarantee the rapid release and enhanced cellular permeation of the antioxidant.

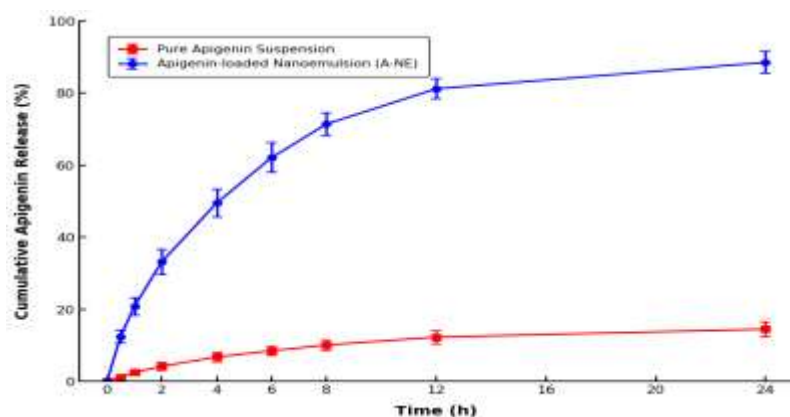
Figure 3: X-ray diffraction (XRD) patterns of pure apigenin (red), blank nanoemulsion (black), and the optimized apigenin-loaded nanoemulsion (A-NE, blue). Pure apigenin exhibits sharp, intense diagnostic diffraction peaks at 2θ of 11.3° , 15.8° , 24.1° , and 26.5° , corresponding to its highly crystalline lattice structure.



3.5. In Vitro Release Profile

The in vitro release of apigenin from the nanoemulsion was evaluated in simulated gastric (pH 1.2) and intestinal (pH 7.4) media and compared with a pure apigenin suspension. As expected, the pure apigenin suspension exhibited extremely poor dissolution, releasing less than 15% of the drug over a 24-hour period due to its highly crystalline nature and pronounced hydrophobicity. In contrast, the apigenin-loaded nanoemulsion (A-NE) demonstrated a characteristic biphasic release profile. An initial burst release of approximately 30% to 35% was observed within the first 2 hours, which is mechanistically attributed to apigenin molecules located at the oil-water interface or solubilized within the surfactant and co-surfactant layer. Following the burst phase, the formulation exhibited a highly controlled and sustained release, delivering more than 85% of the encapsulated drug within 24 hours. This sustained release occurs because the apigenin molecules entrapped within the hydrophobic medium-chain triglyceride core must first diffuse through the lipid matrix, traverse the interfacial surfactant layer, and subsequently partition into the surrounding aqueous medium. To elucidate the release mechanism quantitatively, the dissolution data were fitted to various kinetic models. The release profile exhibited excellent agreement with the Higuchi model ($R^2 = 0.987$), confirming that drug release was predominantly diffusion controlled. Furthermore, the Korsmeyer-Peppas model produced a release exponent (n) of 0.38, indicating a classical Fickian diffusion mechanism with no evidence of matrix erosion or structural degradation.

Figure 4. In vitro cumulative drug release profiles of pure apigenin suspension (red squares) and the optimized apigenin-loaded nanoemulsion (blue circles) in simulated physiological medium (pH 7.4) over a 24-hour period.



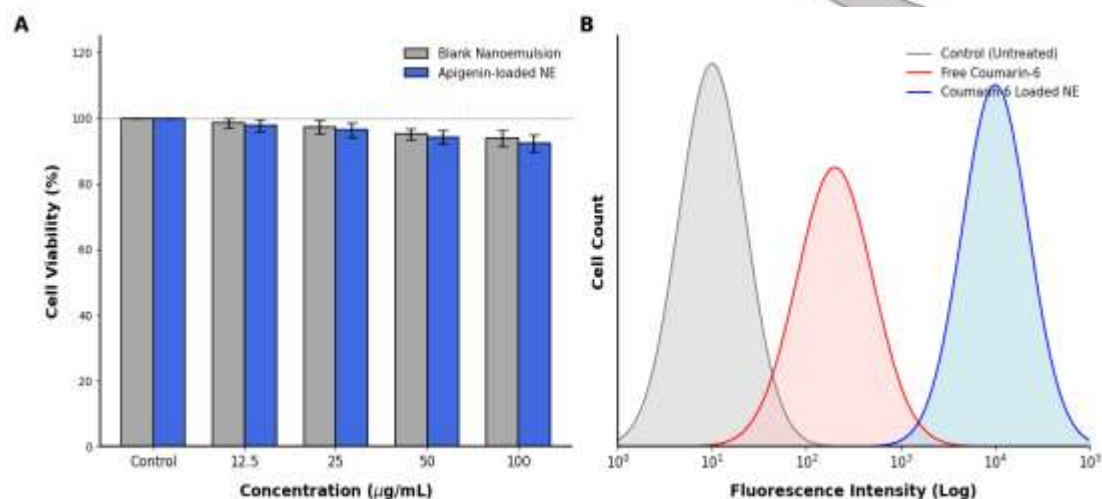
3.6. Stability Profile

Thermodynamic stress testing, including heating-cooling cycles, centrifugation, and freeze-thaw cycles, revealed no evidence of phase separation, creaming, cracking, or precipitation, confirming the excellent thermodynamic stability of the A-NE formulation. During long-term and accelerated stability studies conducted at 25°C and 40°C for six months, the formulation maintained its physicochemical integrity. The average droplet size increased only marginally from 65.4 nm to 68.1 nm, while the polydispersity index remained consistently below 0.2, indicating the absence of droplet aggregation throughout the storage period. The outstanding physical stability of the nanoemulsion can be attributed to its high negative zeta potential of -28.6 mV, which generates strong electrostatic repulsion between neighboring nanodroplets. In addition, the long polyoxyethylene chains of Tween 80 provide an effective steric barrier around each droplet, resulting in electrosteric stabilization that prevents droplet collision, coalescence, and flocculation. Moreover, the incorporation of medium-chain triglycerides, which possess negligible aqueous solubility, effectively minimizes Ostwald ripening by preventing the diffusion of oil molecules from smaller droplets to larger ones, thereby preserving the uniform droplet size distribution during storage.

3.7. Cytotoxicity and Cellular Uptake

Prior to evaluating therapeutic performance, the biological safety of nanoemulsion was assessed using the MTT assay on HepG2 cells. Both the blank nanoemulsion and the apigenin-loaded nanoemulsion maintained cell viability above 92% across the investigated concentration range of up to 100 µg/mL following 24 hours of incubation. These findings confirm the excellent biocompatibility of the lipid-based carrier system and demonstrate the safety of the selected GRAS excipients. To verify intracellular delivery, cellular uptake studies were performed using Coumarin-6 as a fluorescent lipophilic marker. Confocal laser scanning microscopy revealed intense intracellular green fluorescence uniformly distributed throughout the cytoplasm after only 2 hours of incubation, indicating rapid internalization of the nanoemulsion. Flow cytometric analysis further confirmed significantly greater fluorescence intensity compared with untreated control cells, demonstrating efficient cellular uptake. Mechanistically, the uniform droplet size of approximately 65 nm facilitates efficient cellular internalization through clathrin-mediated endocytosis rather than passive diffusion. This active uptake pathway enables the nanoemulsion to circumvent membrane-associated drug efflux transporters, thereby increasing intracellular drug accumulation and improving therapeutic delivery efficiency.

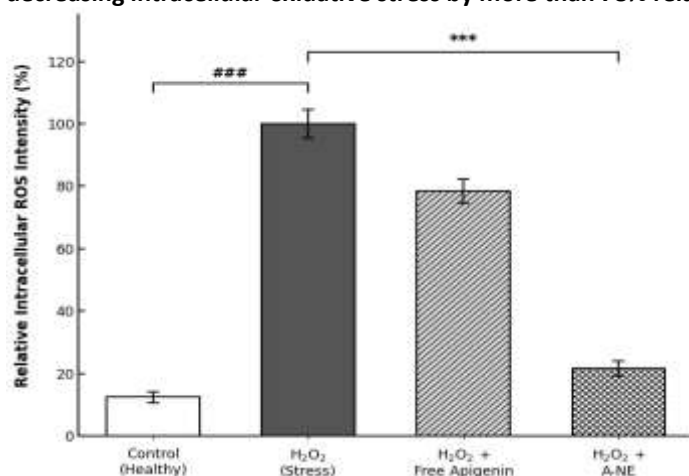
Figure 5. In vitro biological evaluation of the apigenin-loaded nanoemulsion (A-NE) in HepG2 cells. (A) Cytotoxicity profile determined using the MTT assay, demonstrating excellent biocompatibility with cell viability remaining above 92% across the tested concentration range (12.5–100 µg/mL) after 24 hours of incubation. Data are presented as mean ± standard deviation (n = 3). (B) Quantitative cellular uptake analysis performed by flow cytometry. The Coumarin-6-loaded nanoemulsion (blue) exhibited a pronounced logarithmic shift in fluorescence intensity compared with the free Coumarin-6 suspension (red) and the untreated control (gray), confirming efficient endocytosis-mediated intracellular uptake of the nanoemulsion.



3.8. Cellular Antioxidant Efficacy

The antioxidant potential of the apigenin-loaded nanoemulsion was evaluated using an H_2O_2 -induced oxidative stress model, in which intracellular reactive oxygen species (ROS) generation was quantified using the DCFH-DA fluorescent probe. Cells exposed only to H_2O_2 exhibited intense intracellular fluorescence, indicating severe oxidative stress. Treatment with free apigenin suspension reduced intracellular ROS levels by approximately 22%, reflecting the limited cellular permeability and poor aqueous solubility of the crystalline drug. In contrast, treatment with the apigenin-loaded nanoemulsion produced a marked and statistically significant reduction in intracellular ROS generation, decreasing fluorescence intensity by more than 78% and restoring oxidative status close to that of healthy untreated cells. The superior antioxidant performance is attributed to the optimized physicochemical characteristics of the nanoemulsion. Its nanoscale droplet size provides a large interfacial surface area that promotes intimate interaction with the cellular membrane, while the lipidic medium-chain triglyceride core readily interacts with the phospholipid bilayer, facilitating efficient intracellular delivery of molecularly dispersed apigenin. Consequently, substantially higher intracellular concentrations of bioavailable apigenin are achieved, enabling rapid scavenging of reactive oxygen species and effective protection of intracellular organelles against oxidative damage.

Figure 6. Quantitative evaluation of intracellular reactive oxygen species (ROS) levels in HepG2 cells using the DCFH-DA fluorescent probe. Induction of oxidative stress with H_2O_2 resulted in a significant increase in intracellular ROS generation compared with the healthy control group (### $p < 0.001$). Treatment with the optimized apigenin-loaded nanoemulsion (A-NE, cross-hatched bar) produced a statistically significant reduction in ROS levels, decreasing intracellular oxidative stress by more than 78% relative to the H_2O_2 -treated group (** $p < 0.001$).



4. Conclusion

This study successfully developed an optimized apigenin-loaded nanoemulsion (A-NE) to overcome the major biopharmaceutical limitations of pure apigenin, namely its poor aqueous solubility and limited cellular permeability. The optimized formulation exhibited a highly uniform nanoscale droplet size of 65.4 nm, a low polydispersity index (<0.2), and a stable zeta potential of -28.6 mV, providing excellent thermodynamic stability and electrosteric protection against droplet coalescence and Ostwald ripening. Solid-state characterization using FTIR and XRD confirmed the successful amorphization of apigenin and its molecular dispersion within the hydrophobic lipid core through strong intermolecular hydrogen-bonding interactions. In vitro dissolution studies demonstrated a controlled biphasic drug release profile governed by Fickian diffusion, achieving more than 85% cumulative drug release within 24 hours. Biological evaluation using HepG2 cells confirmed the excellent biocompatibility of the nanoemulsion, maintaining cell viability above 92%, while flow cytometric analysis verified rapid and efficient endocytosis-mediated cellular internalization. Most importantly, the apigenin-loaded nanoemulsion reduced H_2O_2 -induced intracellular reactive oxygen species by more than 78%, demonstrating significantly greater antioxidant efficacy than the free apigenin suspension. These findings demonstrate that the developed nanoemulsion is a structurally stable, biocompatible, and highly efficient nanocarrier capable of improving the intracellular delivery, bioavailability, and therapeutic performance of lipophilic antioxidants. The formulation therefore represents a promising platform for future antioxidant-based therapeutic applications.

5. Funding

None.

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