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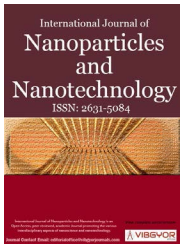
Enhanced Oral Efficacy of Apigenin through Mixed Micelles: *In Vitro* Anti-Tumor Activity and Pharmacokinetic Investigations



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Abstract

Our objective was to design a mixed micelle for increasing the oral bioavailability of apigenin and its *in vitro* anti-tumor activity. Apigenin-loaded mixed micelles were prepared with soluplus and D-α-tocopherol polyethylene glycol 1000 succinate by film dispersion method. The mixed micelles were characterized by determining particle size distribution, drug loading, *in vitro* release, and stability. In addition, the cellular uptake, cytotoxicity, apoptosis and active oxygen generation was determined with Cytation5 cell imaging multifunctional detector and flow cytometry. Finally, in vivo pharmacokinetic in rats were evaluated. The mixed micelles with an average size (62.83 nm) had high encapsulation efficiency (96.41%) and drug loading (4.45%). The *in vitro* drug release profile showed characteristics of sustained release. The *in vitro* experiments showed that the apigenin-loaded mixed micelles improved cellular uptake and cytotoxicity, accelerated cell apoptosis, and elevated ROS levels in MCF-7 and HepG2 cells as compared with the pure apigenin. Correspondingly, the relative oral bioavailability of apigenin in mixed micelles was increased considerably by 4.32-fold in rats compared with pure apigenin.

Keywords

Apigenin, Mixed micelles, Poor water-soluble drug, Anti-tumor activity, Oral bioavailability

Introduction

Oral administration is widely favored for drug delivery due to its convenience and ability to enhance patient compliance. However, the efficacy of this route is often compromised by the harsh environment of the gastrointestinal (GI) tract, where gastric acid, bile, and digestive enzymes can degrade drugs, reducing their absorption efficiency [1]. This challenge necessitates innovative strategies to improve the transport of drugs from the GI tract to the bloodstream, thereby enhancing oral bioavailability.

Apigenin, a naturally occurring flavonoid found abundantly in vegetables and fruits such as parsley, onion, grape, chamomile tea, and red wine [2,3], has garnered interest for its multiple pharmacological properties, including antioxidant, anti-inflammatory, neuroprotective, and anti-tumor effects [4-7]. Notably, apigenin has been shown to suppress the proliferation, migration, and invasion of various tumor cells, including those from breast, lung, and blood cancers, while also promoting cell apoptosis and cell cycle inhibition [8-11]. Despite these promising attributes, the clinical application of apigenin is limited because of

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its poor water solubility, gastrointestinal instability, and low oral bioavailability [12].

To solve these limitations, nanotechnology presents innovative solutions by enhancing the solubility and bioavailability of apigenin through various formulations, including nanocrystals, polymeric micelles, liposomes, nanoparticles, and nanosuspensions. Among these, mixed micelles [13], which exhibit nanosized distribution, offer several advantages such as improved stability, solubility, and drug absorption. Their simple preparation process and low toxicity, without the need for organic solvents, render mixed micelles particularly suitable for oral delivery of apigenin [14,15]. For instance, mixed micelles composed of soluplus and pluronic F127 have significantly increased the solubility of apigenin [16], and TPGS/phospholipid-based mixed micelles have substantially improved its intestinal absorption [17].

Soluplus, an amphiphilic synthetic polymer, forms stable micelles in water thanks to its hydrophilic polyethylene glycol outer shell and hydrophobic polyvinyl caprolactam-vinyl acetate inner core. With a critical micelle concentration (CMC) of 6.44×10^{-8} mol/L [18,19], it effectively encapsulates hydrophobic drugs within its lipophilic core, while its hydrophilic corona ensures micelle dispersion stability [20]. Similarly, TPGS, a non-ionic surfactant and pharmaceutical excipient, enhances drug absorption by inhibiting the efflux pump P-glycoprotein in intestinal epithelial cells and micelle stability, thus promoting the oral uptake of poorly soluble drugs [21,22].

This study aimed to develop a mixed micelle formulation combining soluplus and TPGS to increase the aqueous solubility, improve the *in vitro* anti-tumor efficacy, and enhance the oral absorption of apigenin. We investigated the physicochemical properties of the apigenin-loaded mixed micelles, including particle size, zeta potential, *in vitro* release profile, storage stability, and Fourier-transform infrared spectroscopy (FTIR) analysis. The efficacy of the mixed micelles was further assessed through *in vitro* studies on cell uptake, cytotoxicity, apoptosis, and active oxygen generation in MCF-7 and HepG2 cell lines. Finally, the pharmacokinetic behavior of apigenin-loaded mixed micelles in rats was evaluated to ascertain their potential as an effective oral therapeutic agent.

Materials and Methods

Materials

Apigenin (98% purity), coumarin 6, and D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) were obtained from Sigma-Aldrich (USA). Soluplus was provided by BASF Auxiliary Chem. Co., Ltd. (Shanghai, China). Methanol of high-pressure liquid chromatography (HPLC) grade was purchased from Fisher Scientific (Beijing, China), and all other chemicals were of analytical grade. Water purified to Milli-Q grade by a Millipore system (ELGA Lab Water, Sartorius, UK) was used throughout the research.

The human breast cancer cell line MCF-7 and the human hepatocellular carcinoma cell line HepG2 were sourced from the Basic Laboratory of Guangdong Medical University. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C in a 5% CO_2 atmosphere in a humidified incubator (Japan Panasonic Sanyo, Co, Ltd, Japan).

Female Sprague-Dawley rats (weight: 200 ± 20g) were provided by the Guangdong Medical Laboratory Animal Center (China). The animals were acclimated in cages for two weeks with access to food and water freely. Before the commencement of experiments, the rats were fasted for 12 hours but allowed free access to water. All procedures were approved by the Institute's Animal Care and Use Committee at Guangdong Medical University (approval No. 2023-0099).

Preparation of Apigenin-loaded mixed micelles

Apigenin-loaded mixed micelles were prepared using a slightly modified thin-film dispersion method [23]. A precise quantity of apigenin and soluplus were dissolved in ethanol, and the solvent was subsequently evaporated at 37 °C using a vacuum rotary evaporator to form a thin polymeric film inside a 50-mL round-bottom flask. The thin film was further dried under vacuum overnight. The resultant dry polymeric film was rehydrated with a specific volume of TPGS aqueous solution and stirred magnetically for 2 hours to form the apigenin-loaded mixed micelles. To remove any undissolved drugs, the mixed micelles were achieved by filtrating through a 0.22 μm polycarbonate membrane, yielding a clear supernatant as the micelle solution.

Characterization of Apigenin-loaded mixed micelles

Particle size and zeta potential determination: The particle size distribution and zeta potential of the apigenin-loaded mixed micelles were measured at room temperature using Zetasizer Nano ZS-90 (Malvern, UK). To achieve a suitable concentration for analysis, samples were diluted with deionized water. This measurement was conducted in triplicate under consistent conditions.

Encapsulation efficiency and drug loading determination: To quantify the drug loading (DL) content and encapsulation efficiency (EE) of micelles, the micelles were dissolved in methanol, disrupting the nanostructures [24]. The apigenin concentration was then measured using an ultraviolet-visible spectrophotometer (UV-650, Shanghai Meipuda Instrument Co. Ltd, China) at a wavelength of 336 nm. The EE and DL were calculated according to the formulas: EE (%) = (W_a/W_{total}) × 100%, and DL (%) = (W_a/W_{MMs}) × 100%, where W_a is the weight of the drug in the micelles, W_{total} is the total weight of the drug used, and W_{MMs} is the combined weight of the drug and the nanocarriers.

***In vitro* release:** The *in vitro* release profile of apigenin from the mixed micelles was examined using the dialysis bag method (MWCO 14000 Da, Solarbio, China) [24]. For this experiment, 0.5 mL of apigenin-loaded mixed micelles (containing 100 µg of apigenin) were sealed in a dialysis bag, which was then submerged in 20 mL of pH 1.2 release medium for 2 hours, followed by immersion in pH 7.4 release medium for the subsequent 46 hours. The experiment was conducted at (37 ± 0.5) °C with a stirring speed of 100 rpm. At predetermined intervals, all release medium was sampled and replaced with an equal volume of fresh medium. The apigenin suspension served as controls, and the drug content was measured using UV spectroscopy at a wavelength of 336 nm. This procedure was repeated three times. To analyze the mechanism of apigenin release from the mixed micelles, the release data were fitted to the following kinetic models:

Zero-order model equation: $y = k_1t + a_1$

First-order model equation: $\ln (100-y) = k_2t + a_2$

Higuchi's square-root equation: $y = k_3t^{0.5} + a_3$

Here, y represents the cumulative percentage of apigenin released, t is the sampling time, k_1, k_2, and k_3 are the rate constants for the zero-order, first-order, and Higuchi models, respectively, and a_1 to a_3 are constants associated with each model.

Fourier Transform Infrared Spectroscopy (FTIR)

The interactions between drugs and carriers were examined using FTIR spectroscopy. The FTIR spectra of pure apigenin, blank mixed micelles, a physical mixture of blank mixed micelles with pure apigenin, and apigenin-loaded mixed micelles were obtained using a WQF-510A Fourier Transform Infrared Spectrometer (Beijing Rayleigh Analytical Instruments Co., Ltd, Beijing, China) equipped with a Smart OMNI-sampler accessory. Samples of their dry powder were thoroughly samples with an appropriate amount of KBr and then compressed into pellets using a hydraulic press. These pellets were subjected to infrared radiation, and their spectra were recorded across the range of 400 to 4,000 cm^{-1}.

Stability study

The physical stability of the apigenin-loaded mixed micelles was assessed by storing their dispersion in glass vials at 4 °C for one month. The particle size and encapsulation efficiency were evaluated at intervals of 0, 1, 2, 3, and 4 weeks after preparation.

Cellular uptake

The cellular uptake of the micelles was analyzed in MCF-7 and HepG2 cells both qualitatively, using Cytation5 cell imaging multifunctional detector, and quantitatively, through flow cytometry. Initially, cells were seeded into 6-well plates at a density of approximately 5×10^4 cells per well and allowed to culture for 24 hours. The cells were then treated with pure coumarin 6, coumarin 6-loaded micelles, and coumarin 6-loaded mixed micelles for 4 hours. The concentration of coumarin 6 in each treatment was 0.1 µM, with blank medium serving as the control. For the quantitative analysis, after treatment, cells were washed thrice with cold PBS, fixed with 4% paraformaldehyde for 10 minutes, and the cell nuclei were stained with Hoechst 33342. Observations were made using Cytation5 cell imaging multifunctional detector with an excitation wavelength of 488 nm and an emission wavelength

of 560 nm. For quantitative assessment, cells were trypsinized, washed with ice-cold PBS, and collected. The collected cells were resuspended in 0.3 mL of binding buffer and analyzed by flow cytometry to determine the fluorescence intensity of coumarin 6 (excitation wavelength = 488 nm, emission wavelength = 560 nm).

In vitro cytotoxicity

Cytotoxicity was assessed using the sulforhodamine B (SRB) staining assay [25]. MCF-7 and HepG2 cells were seeded at a density of 5×10^3 cells/well into 96-well culture plates and cultured for 24 hours in a 5% CO_2 atmosphere at 37 °C. Upon reaching approximately 80% confluence, cells were treated with pure apigenin, apigenin-loaded micelles, and apigenin-loaded mixed micelles, respectively, with apigenin concentrations ranging from 0.01 to 10 μM. Blank mixed micelles served as parallel controls, and blank medium was used as a blank control. After 48 and 72 hours of incubation, cells were fixed with trichloroacetic acid, rinsed with deionized water, and stained with SRB. Absorbance was measured at 540 nm using Cytation5 cell imaging multifunctional detector. Cell survival rates were calculated with the formula: survival rate % = (OD_{540} nm of treated cells/OD_{540} nm of control cells) × 100%, where OD_{540} nm represents absorbance at 540 nm.

Apoptosis

Apoptosis in MCF-7 and HepG2 cells was evaluated using both qualitative and quantitative methods. Cells were plated at a density of 5×10^4 cells per well in 6-well plates and cultured for 24 hours. Then, cells were treated with pure apigenin, apigenin-loaded micelles, and apigenin-loaded mixed micelles for 36 hours, with apigenin at a final concentration of 5 μM. Blank medium was used as a control. For qualitative observations, cells were stained with Hoechst 33342, and cellular and nuclear morphology were observed using Cytation5 cell imaging multifunctional detector. For quantitative assessment, cells were trypsinized, washed with ice-cold PBS, collected, and stained with Annexin V647/7-AAD for 10 minutes at room temperature in the dark. After adding binding buffer, samples were analyzed by flow cytometry to determine the rate of apoptosis induced.

Cell active oxygen

Active oxygen levels were measured by flow cytometry. Cells were seeded at a density of 2×10^5 cells per well in 6-well plates and cultured for 24 hours. After removing the culture medium, cells were treated with pure apigenin, apigenin-loaded micelles, and apigenin-loaded mixed micells, with the final concentration of apigenin being 5 μM. Blank medium was used as a control. After incubation for 36 hours, cells were exposed to the fluorescent probe DCFH-DA, diluted to a final concentration of 10 μM in serum-free medium, and incubated for 25 minutes at 37 °C. Cells were then washed, trypsinized with 0.25% Trypsin-EDTA, and collected. The collected cells were resuspended in 0.3 mL of binding buffer and analyzed by flow cytometry.

In vivo pharmacokinetics study

Rats were randomly divided into two groups each made up of five rats and orally administered with apigenin-loaded mixed micelles and pure apigenin at equivalent concentrations. The apigenin suspension was dispersed in distilled water containing 0.3% (v/v) CMC-Na, with the apigenin dose maintained at 60 mg/kg [26]. Blood samples were collected from the retro-orbital plexus of rats at predetermined times (0.0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48, and 72.0 hours), placed into heparinized tubes, immediately centrifuged (5000 rpm for 10 min), and the separated plasma was stored at -20 °C until analysis.

Standard samples were prepared by mixing appropriate volumes of drug solution with drug-free plasma, establishing a standard curve for apigenin within the concentration range of 0.10 ~ 6.00 μg/mL ($R^2 = 0.9591$). The method's reliability was verified by estimating the precision, accuracy, recovery, and limit of quantification.

To extract and analyze compounds from plasma, a 100 μL sample was mixed with 1 mL of methanol, serving both as the extraction solvent and protein precipitant. This mixture was vortexed for 3 minutes and then centrifuged at 12,000 rpm for 15 minutes at room temperature. After centrifugation, the supernatant was evaporated under a nitrogen atmosphere. The residue was redissolved in 100 μL of methanol and centrifuged once more under the same conditions. An 80 μL aliquot of the final solution was injected into an HPLC system, which was equipped with a guard column. The mobile phase consisted of methanol and water in a 60:40 v/v ratio, with a flow rate of 1.0 mL/min. Detection

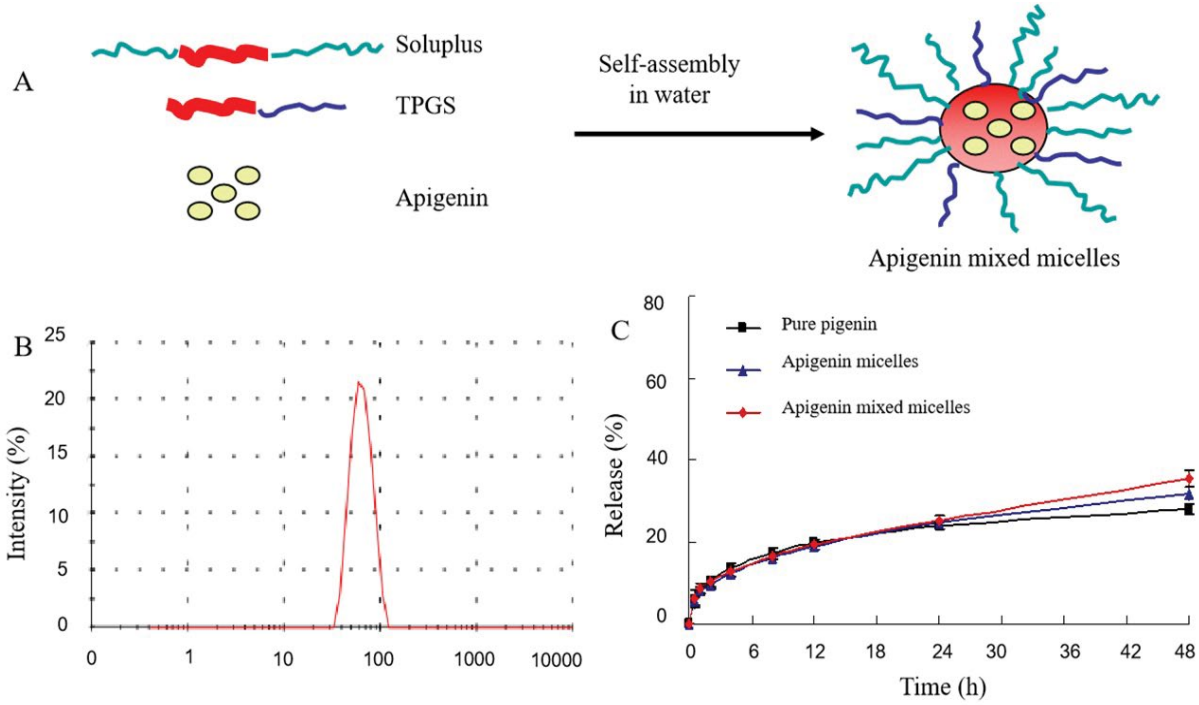

Figure 1: Characterizations of apigenin-loaded mixed micelles. A) Diagrammatic sketch of forming procedure; B) Particle size distribution; C) Apigenin release rates (%) of apigenin solution, apigenin-loaded micelles, and apigenin-loaded mixed micelles. Data are presented as mean ± SD (n = 3).

Table 1: Characterization of micelles (mean ± SD, n = 3).

Sample	size (nm)	PDI	Zeta (mV)	EE (%)	DL (%)
Blank mixed micelles	59.35 ± 1.07	0.09 ± 0.02	-8.65 ± 0.94	-	-
Apigenin-loaded micelles	61.93 ± 0.91	0.08 ± 0.01	-10.0 ± 1.22	93.68 ± 3.61	4.58 ± 0.16
Apigenin-loaded mixed micelles	62.38 ± 1.13	0.09 ± 0.01	-16.5 ± 2.31	96.41 ± 3.07	4.45 ± 0.11

was performed at a wavelength of 336 nm, and pharmacokinetic parameters were subsequently calculated using DAS software (version 2.0).

Statistical analysis

One-way analysis of variance (ANOVA) was used for the initial analysis, followed by post-hoc tests with bonferroni correction for multiple comparisons. Data were reported as means ± standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

Results

Characterization of Apigenin-loaded mixed micelles

Figure 1A showed a schematic diagram of micelle formation. The mean particle size, zeta potential and PDI of mixed micelles were measured by Zetasizer Nano ZS-90 (Malvern, UK), and the data were showed in Table 1. The average diameters of the apigenin-loaded mixed micelles were found to be 62.38 nm (Figure 1B), indicating a uniform particle size distribution with a polydispersity index of less than 0.1. This uniformity suggested that the particles were homogeneously sized. The zeta potential analysis revealed that apigenin-loaded mixed micelles were negatively charged, with values of - (16.5 ± 2.3) mV, demonstrating the micelles' stability. The encapsulation efficiency (EE) and drug loading (DL) for the apigenin-loaded mixed micelles were recorded at 96.41% and 4.45%, respectively, indicating efficient encapsulation and compatibility between the drug and the carriers.

Releases *in vitro*

In vitro release profiles of apigenin from different formulations were analyzed under simulated gastric (pH 1.2) and intestinal (pH 7.4) conditions (Figure 1C). The cumulative release of apigenin from the micelles showed approximately

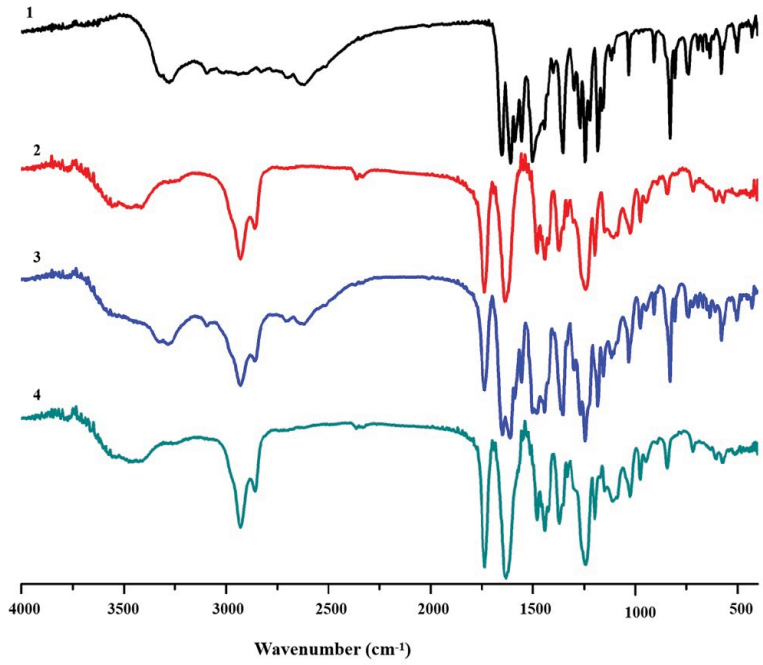

Figure 2: Infrared spectrum. 1, pure apigenin; 2, blank mixed micelles; 3, physical mixture of blank mixed micelles and apigenin; 4, apigenin-loaded mixed micelles.

Table 2: Different release model fitting equations for apigenin-loaded mixed micelles.

Kinetic equation	Regression equation	R^2
Zero-order	$Q = 0.498^{*}t + 10.820$	0.9777
First-order	$Q = 100(1-e^{-0.009^{*}t})$	0.9899
Higuchi	$Q = 5.326^{*}t^{1/2}$	0.9991

7.52% release in pH 1.2 within the first 2 hours, and 35.72% in pH 7.4 over 48 hours for apigenin-loaded micelles. For apigenin-loaded mixed micelles, the release was about 5.71% in pH 1.2 within 2 hours, and 41.51% in pH 7.4 over 48 hours. The apigenin suspension released 15.35% in pH 1.2 within 2 hours and 29.83% in pH 7.4 over 48 hours. The release kinetics of apigenin-loaded mixed micelles were best described by Higuchi's equation, with a linear relationship ($R^2 > 0.99$) between release rate and the square root of time, suggesting diffusion-driven release (Table 2).

Fourier Transform Infrared Spectroscopy (FTIR)

Figure 2 showed the result of FTIR. FTIR analysis showed characteristic absorption bands of apigenin at hydroxyl stretching bands (3278 cm^{-1}), carbonyl stretching bands (1654 cm^{-1}), and carbon-carbon double bands (1608 cm^{-1}). Blank mixed micelles displayed carbonyl stretching bands at 1737 cm^{-1} and 1639 cm^{-1}, and a hydrocarbon stretching band at 2930 cm^{-1}. In the physical mixtures of blank mixed micelles with apigenin, the characteristic bands of both components remained unchanged, indicating no significant interaction between them. However, in the apigenin-loaded mixed micelles, the hydroxyl stretching peaks of apigenin shifted to 3500 cm^{-1} and broadened, and the carbonyl stretching bands absorption peaks of the carrier shifted to 1735 cm^{-1} and 1631 cm^{-1}. These spectral changes suggested the formation of intermolecular hydrogen bonds between the hydroxyl groups of apigenin and the carbonyl groups in the carrier [20].

Storage stability

The storage stability of apigenin-loaded mixed micelles was evaluated over a month at 4 °C. The entrapment efficiency and particle size were monitored weekly, as shown in Figure 3. Throughout the storage period, no significant alterations in entrapment efficiency or particle size were observed, indicating that apigenin-loaded mixed micelles remained stable under these storage conditions.

Cellular uptake

The cellular uptake rate, a critical determinant of therapeutic efficacy, was assessed qualitatively and quantitatively in MCF-7 and HepG2 cells

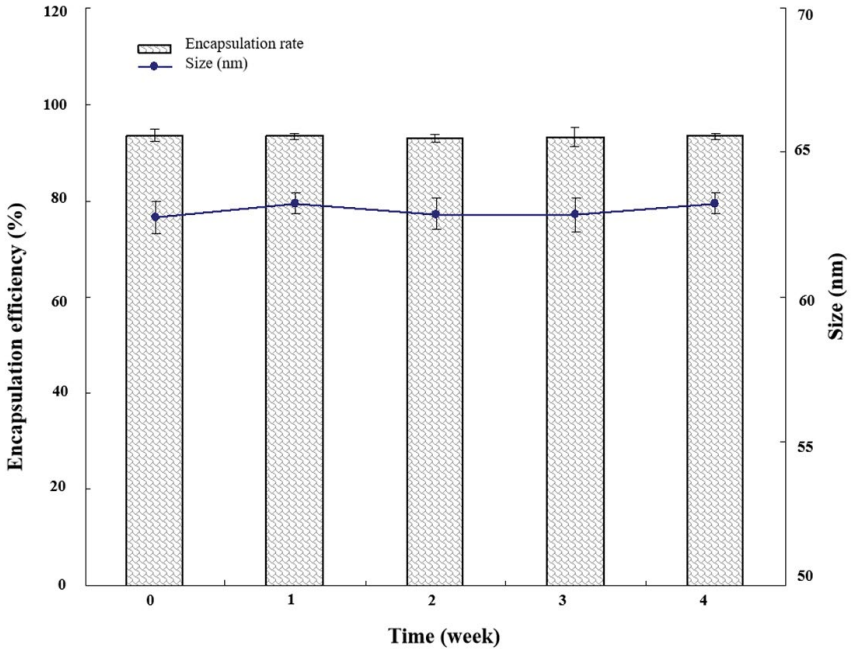

Figure 3: The storage stability of apigenin-loaded mixed micelles in vitro. Data are presented as mean ± SD (n = 3).

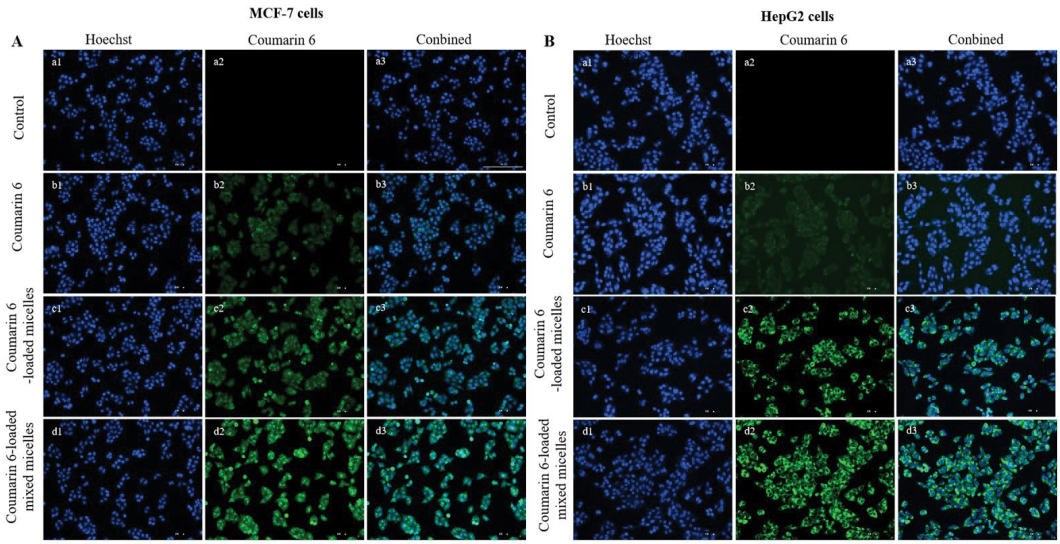

Figure 4: Intracellular uptake by MCF-7 cells (A) and HepG2 cells (B) after treatment with varying formulations by Cytation5 cell imaging multifunctional detector.

using the cytation5 cell imaging multifunctional detector and flow cytometry. Coumarin 6 served as the fluorescent probe for these evaluations. Intracellular drug levels in MCF-7 and HepG2 cells, depicted in Figure 4, showed green fluorescence in the cytoplasm after treatment with pure coumarin 6, coumarin 6 -loaded micelles, and coumarin 6-loaded mixed micelles, indicating successful cellular uptake. Notably, cells treated with coumarin 6-loaded mixed micelles displayed significantly stronger fluorescence than those treated with pure coumarin 6.

Figure 5 presented the cellular uptake levels post-incubation with various apigenin formulations. Intracellular coumarin 6 levels were measured as (750 ± 137.18), (1110.5 ± 102.53), and (1269.5 ± 89.8) in MCF-7 cells and (1449 ± 94.75), (2370 ± 25.46), and (2624.5 ± 70.01) in HepG2 cells for pure coumarin 6, coumarin 6-loaded micelles, and coumarin 6-loaded mixed micelles, respectively.

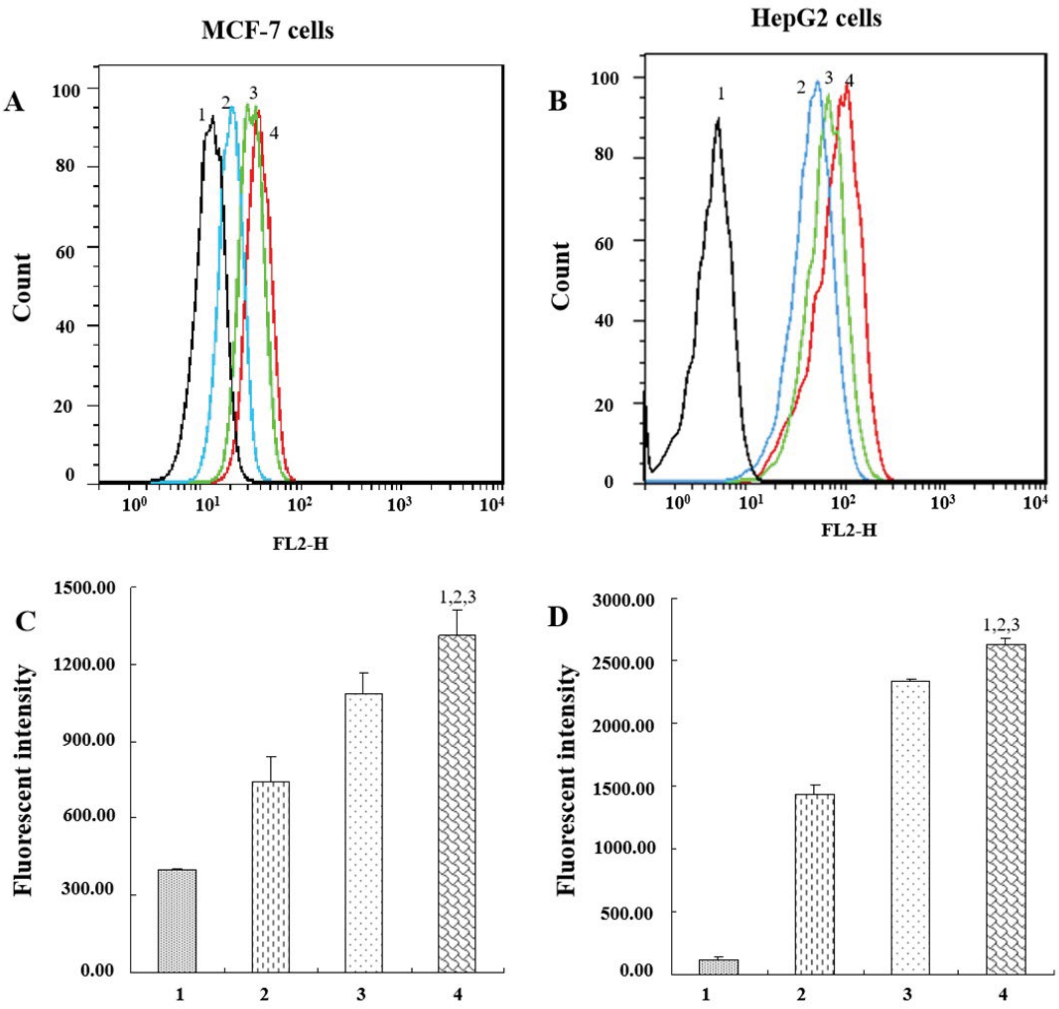

Figure 5: Intracellular uptake by MCF-7 cells (A) and HepG2 cells (B) after treating with varying formulations by flow cytometry. 1, control; 2, pure coumarin 6; 3, coumarin 6-loaded micelles; 4, coumarin 6-loaded mixed micelles, $P < 0.05$. Data are presented as mean ± SD (n = 3).

The uptake of coumarin 6 from mixed micelles was roughly twice that from pure coumarin 6.

In vitro cytotoxicity

The SRB assay was utilized to evaluate the cytotoxicity of apigenin-loaded mixed micelles compared with pure apigenin and apigenin-loaded micelles. Figure 6 illustrated the dose-dependent and time-dependent inhibitory effects on cell proliferation of MCF-7 and HepG2 cells treated with different apigenin formulations. The half-maximal inhibitory concentration (IC50) value on MCF-7 cells or HepG2 cells was listed in Table 3. Significant differences in cell viability were observed among treatments with pure apigenin, apigenin-loaded micelles, and apigenin-loaded mixed micelles, with the mixed micelles and micelles both showing enhanced cytotoxic effects over pure apigenin. The cytotoxicity sequence was apigenin-loaded mixed micelles > apigenin-loaded micelles > pure apigenin, aligning with the cellular uptake findings. Blank mixed micelles, tested at 800 μg/mL, showed no significant cytotoxicity to MCF-7 and HepG2 cells with over 98.82 ± 5.78% cell viability for 48 hours, indicating their safety.

Apoptosis-inducing effect

Apoptosis was qualitatively and quantitatively analyzed in MCF-7 and HepG2 cells using the cytation5 cell imaging multifunctional detector and flow cytometry. Exposed cells exhibited apoptotic features such as nuclear condensation and separation, cytoplasmic condensation, and membrane folds (Figure 7A and Figure 7B), with apigenin-loaded mixed micelles inducing higher apoptosis rates than other formulations.

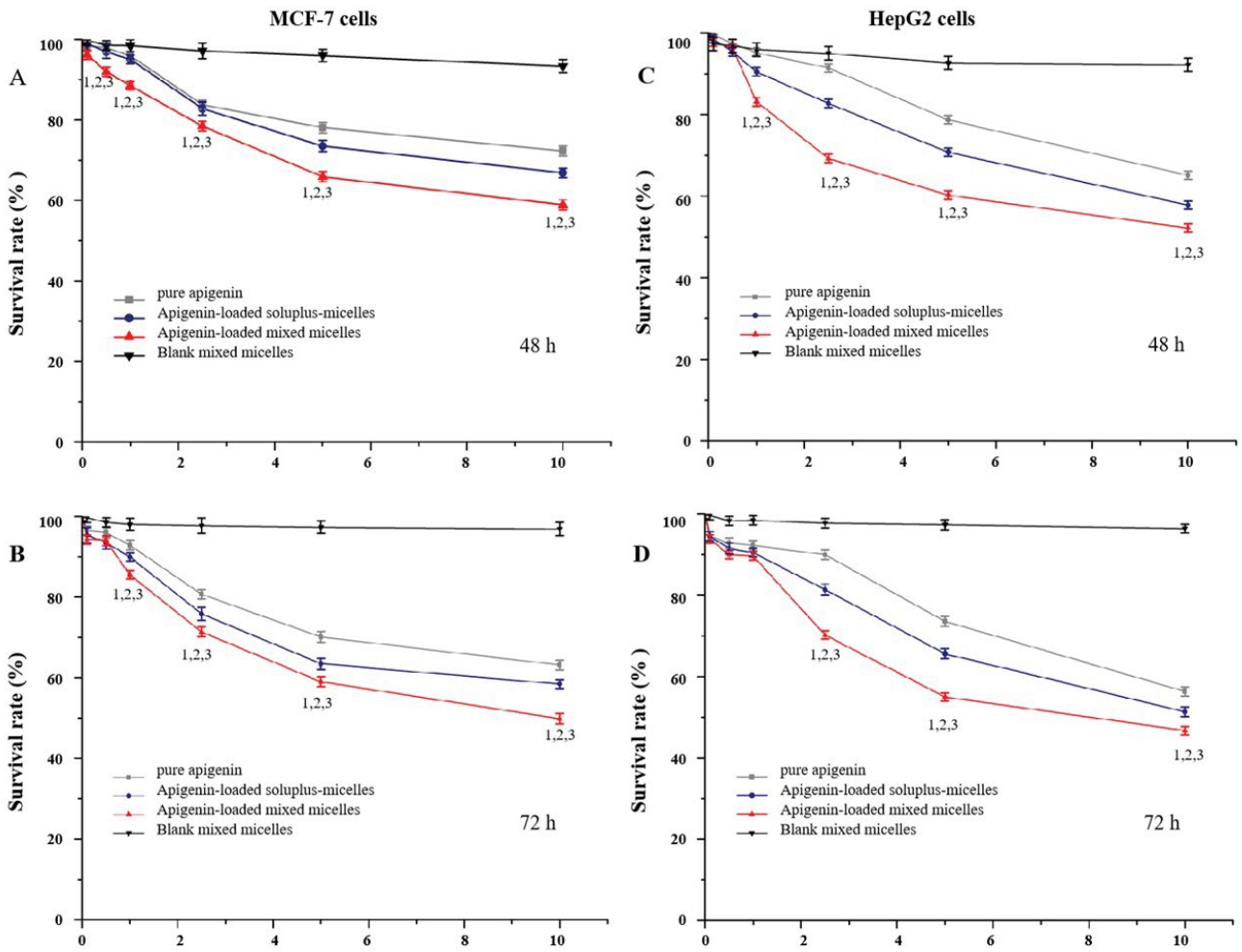

Figure 6: Survival rates (%) of MCF-7 cells (A, B) or HepG2 cells (C, D) after treating with varying formulations for 48 hours or 72 hours. 1, blank mixed micelles; 2, pure apigenin; 3, apigenin-loaded micelles; 4, apigenin-loaded mixed micelles, $P < 0.05$. Data are presented as mean ± SD (n = 3).

Table 3: IC50 (μM) of pure apigenin, apigenin-loaded micelles, and apigenin-loaded mixed micelles after 48 hours, and 72 hours incubation with MCF-7 cells or HepG2 cells (mean ± SD, n = 3).

Sample	MCF-7 cells		HepG2 cells	
	48 h	72 h	48 h	72 h
Pure apigenin	27.85 ± 4.15	19.1 ± 3.31	18.26 ± 2.85	15.31 ± 2.59
Apigenin-loaded micelles	23.43 ± 3.51	13.14 ± 2.74	14.04 ± 1.63	12.16 ± 1.62
Apigenin-loaded mixed micelles	17.62 ± 3.17	9.66 ± 1.04	9.93 ± 1.15	7.86 ± 1.37

Notes: IC_{50}: Half-maximal inhibitory concentration; SD: Standard Deviation

Figure 7C and Figure 7D presented the apoptosis rates post-incubation with various apigenin formulations stained with FITC Annexin V 647/7AAD to detect apoptosis and necrosis. The apoptosis rates were (4.75 ± 0.45)% in MCF-7 cells and (4.85 ± 0.36)% in HepG2 cells for pure apigenin. However, the apoptosis rates were (6.7 ± 0.5)% and (13.2 ± 0.5)% in MCF-7 cells and (7.55 ± 0.65)% and (14.25 ± 0.25)% in HepG2 cells for apigenin-loaded micelles and apigenin-loaded mixed micelles. Therefore, at equivalent drug concentrations, the order of apoptosis induction was apigenin-loaded mixed micelles > apigenin-loaded micelles > pure apigenin. The results revealed that apigenin-loaded mixed micelles induced higher apoptosis rates in MCF-7 and HepG2 cells than either apigenin-

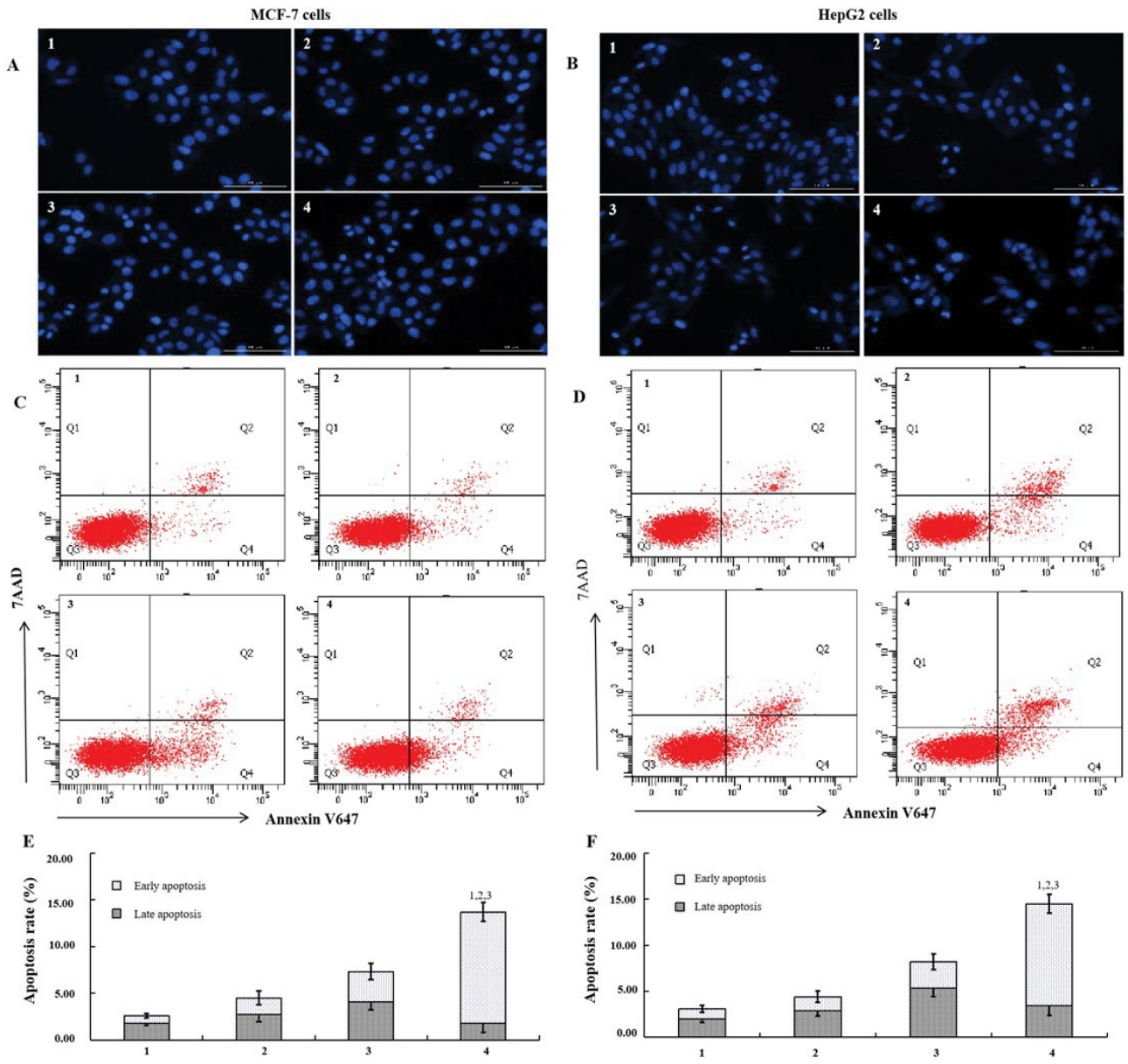

Figure 7: Induced apoptosis on MCF-7 cells or HepG2 cells after treating with varying formulations by Cytation5 cell imaging multifunctional detector (A, B) and by flow cytometry (C, D) 1, control; 2, pure apigenin; 3, apigenin-loaded micelles; 4, apigenin-loaded mixed micelles, $P < 0.05$. Data are presented as mean ± SD (n = 3).

loaded micelles or pure apigenin, corroborating the cytotoxicity results.

Cell active oxygen

Flow cytometry was utilized to measure the reactive oxygen species (ROS) levels in MCF-7 and HepG2 cells treated with various formulations, as illustrated in Figure 8. The ROS levels recorded were 346.5 ± 51.62, 444 ± 15.56, 592.5 ± 9.19, and 799.5 ± 13.44 for MCF-7 cells, and 452 ± 26.87, 661.5 ± 31.82, 863.5 ± 11.31, and 1216.5 ± 23.33 for HepG2 cells, corresponding to treatments with culture medium, pure apigenin, apigenin-loaded micelles, and apigenin-loaded mixed micelles, respectively. These findings indicate that apigenin-loaded mixed micelles significantly increased ROS levels in both MCF-7 and HepG2 cells in comparison to the other treatments. The augmentation in ROS levels is consistent with the enhancement of apoptosis induction, hinting at a potential mechanism through which apigenin-loaded mixed micelles amplify cytotoxicity.

In vivo pharmacokinetics

The oral bioavailability of apigenin-loaded mixed micelles was compared to pure apigenin in rats. The

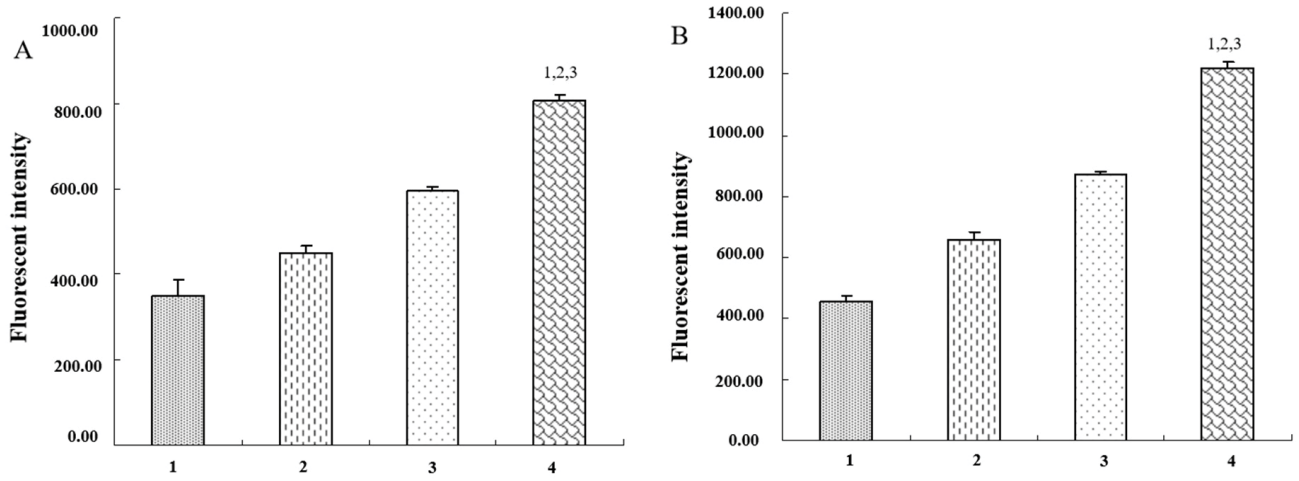

Figure 8: Cellular active oxygen after treating with varying formulations by flow cytometry. 1, control; 2, pure apigenin; 3, apigenin-loaded micelles; 4, apigenin-loaded mixed micelles, $P < 0.05$. Data are presented as mean ± SD (n = 3).

Table 4: Pharmacokinetic parameters of apigenin following oral administration of a single dose (60 mg/kg) of pure apigenin and apigenin-loaded mixed micelles (mean ± SD, n = 6).

PK parameter	Pure apigenin	Apigenin-loaded mixed micelles
C_{max} (μg /mL)	2.32 ± 1.05	2.55 ± 1.27
T_{max} (h)	1.14 ± 0.95	6.03 ± 1.25
MRT (h)	6.48 ± 1.58	16.53 ± 4.82
$t_{1/2}$ (h)	1.44 ± 0.76	3.28 ± 1.51
$AUC_{(0\rightarrow\infty)}$ (μg.h/mL)	14.94 ± 6.75	64.32 ± 17.43

Notes: C_{max}: Peak plasma concentration; T_{max}: Time to reach peak plasma concentration; MRT: Maximum Residence Time; $T_{1/2}$: Elimination half-life; $AUC_{(0\rightarrow\infty)}$: Area under the plasma concentration time curve calculated by the linear trapezoidal rule from time 0 to infinity; SD: Standard Deviation

pharmacokinetic parameters were presented in Table 4 and the plasma concentration-time profiles of apigenin were depicted in Figure 9. These results indicated that apigenin-loaded mixed micelles were released sustainably over 72 hours, whereas pure apigenin was rapidly cleared from circulation within 24 hours. The half-life ($T_{1/2}$) of apigenin in mixed micelles was twice as long as that of the apigenin suspension, and the area under the curve ($AUC_{0\rightarrow\infty}$) of apigenin in mixed micelles was 4.32 times higher than that for the apigenin suspension. Thus, apigenin-loaded mixed micelles exhibited a relative oral bioavailability of 432%, highlighting their potential to enhance drug delivery orally.

Discussion

Polymeric micelle-based drug delivery systems have garnered significant interest for their ability to enhance the absorption of drugs by shielding them from the gastrointestinal (GI) tract environment, thereby improving the bioavailability and delivery of drugs with poor water solubility [27]. Nanomicells form structures with a hydrophobic core and a hydrophilic shell, optimizing the delivery of hydrophobic drugs [28]. The encapsulation of such drugs within the core safeguards them against degradation, thereby increasing their bioavailability. Furthermore, the micelles' diminutive size prolongs their circulation time and promotes better biodistribution [29]. In this study, apigenin-loaded mixed micelles were formulated and demonstrated to improve cellular uptake, cytotoxicity, ROS levels, apoptosis induction, and oral absorption significantly.

The particle size of mixed micelles plays a crucial role in their absorption rate in the GI tract for oral administration. Nanoparticles within the 10-100 nm range are known to exhibit optimal

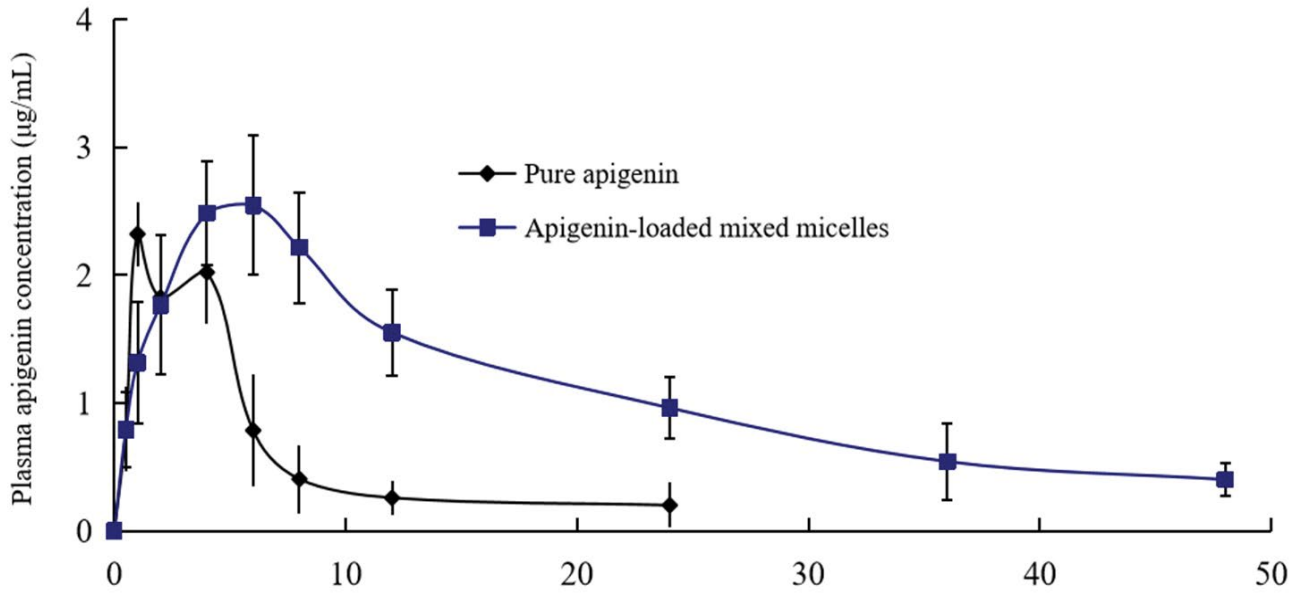

Figure 9: Mean apigenin plasma profiles following a single dose. Data are presented as mean ± SD (n = 5).

uptake in epithelial and smooth muscle cells [30]. Consequently, the ~63 nm particle size of apigenin-loaded mixed micelles contributed to their evasion from rapid metabolism and clearance, ensuring enhanced stability, prolonged circulation time, and improved targeting efficacy due to increased permeability and retention.

The zeta potential is critical for the storage stability of colloidal dispersion systems, with particle aggregation more likely if the zeta potential is too low to offer adequate electrical repulsion or steric barriers [31]. The apigenin-loaded mixed micelles exhibited a zeta potential slightly more negative than that of blank micelles, attributed to the dissociation of acidic phenolic hydroxyl groups in apigenin [32]. Bonilla, et al. prepared apigenin-loaded lipid nanoparticles with a zeta potential of -20 mV, where a higher zeta potential value indicated increased electrostatic repulsion and, hence, greater particle stability [33].

The *in vitro* release studies revealed that apigenin-loaded mixed micelles offered a sustained release profile, with release kinetics adhering to the Higuchi equation, signifying that diffusion was the predominant mechanism of drug release. The sustained release of apigenin from mixed micelles was likely due to hydrogen bonding between the drug and carrier molecules, which slowed the release. These hydrogen bonds not only modulated the drug's release but also enhanced the micelles' stability.

The effectiveness of chemotherapy treatment significantly relies on the cellular uptake of the therapeutic drug. Enhanced cellular drug levels are beneficial for chemotherapy by improving its therapeutic effects and minimizing side effects. In this study, several factors contributed to the increased cellular uptake. Firstly, the mixed micelles could be internalized into cells through nonspecific mechanisms such as phagocytosis or endocytosis [34]. Secondly, the encapsulation of hydrophobic drugs within the micelles' core enhanced their water solubility. Additionally, the sustained release system of mixed micelles maintained higher drug concentrations around the cells. Finally, TPGS, serving as an absorption enhancer, further facilitated this uptake [21]. These findings suggested that TPGS presence could elevate intracellular drug levels, aligning with previous research [21].

The *in vitro* cytotoxicity study revealed that both apigenin-loaded micelles and apigenin-loaded mixed micelles exhibited lower IC50 values compared to pure apigenin, indicating enhanced cytotoxicity effects. The encapsulation efficiency of apigenin in mixed micelles was crucial for this improved cytotoxicity, leading to high local drug concentrations. Moreover, the slow release of apigenin from the micelles prolonged cellular exposure and increased intracellular uptake, contributing to their enhanced cytotoxicity. The

presence of TPGS in the apigenin-loaded mixed micelles likely improved cellular uptake, further boosting their cytotoxic effect, which correlated with the higher cellular uptake rate of tumor cells.

Apoptosis induction is crucially dependent on high intracellular drug concentrations. Thus, micelles' capability to induce apoptosis surpassed that of free drugs. Additionally, the drug release rate from micelles might limit the rate of cellular apoptosis. Unlike free drugs, which were rapidly released and eliminated upon entering cells, drugs from micelles were released slowly, maintaining high concentrations over extended periods. In the case of apigenin-loaded mixed micelles, efficient intracellular transport facilitated by TPGS, which inhibits P-gp, leads to elevated apigenin levels and maximizes apoptotic cell numbers. The ROS results indicated that apigenin-loaded mixed micelles significantly raised ROS levels and induced cell apoptosis.

The *in vivo* pharmacokinetics study aimed to evaluate the relative oral bioavailability of apigenin. Apigenin-loaded mixed micelles achieved significantly higher total plasma concentrations of apigenin compared to the apigenin suspension, positively affecting the therapeutic index. The maximum concentration (C_{max}) of apigenin from mixed micelles surpassed that from the suspension, suggesting enhanced GI tract absorption. The prolonged time to reach maximum concentration (T_{max}) in rats, extended 4-fold, might be attributed to the longer circulation time. This difference *in vivo* behavior between apigenin suspension and mixed micelles was notable. The hydrophobic nature of apigenin in suspension form hindered its absorption by mucus, leading to lower blood accumulation and C_{max}. In contrast, mixed micelles improved drug solubility and stability in the GI tract, with their optimal size (~63 nm) facilitating oral drug absorption. The ideal size range (10-100 nm) of nanomicelles enhanced oral bioavailability by improving endothelial cell membrane transport and absorption into the bloodstream [25]. Moreover, the TPGS component in mixed micelles might inhibit P-gp transporters in enterocytes, preventing apigenin efflux [21]. Consequently, mixed micelles significantly increased apigenin's total plasma concentration and markedly improved its relative oral bioavailability.

Conclusions

In conclusion, apigenin-loaded mixed micelles, formulated with soluplus and TPGS copolymers for oral administration were developed. The apigenin-loaded mixed micelles, with an average size of ~63 nm, exhibited high stability and a controlled release behavior. They showed high cellular uptake and cytotoxicity against cancer cells, effectively inducing apoptosis and elevating ROS levels. The findings suggested that the mixed micelles represented a potential candidate to boost the cytotoxic effects and the relative oral bioavailability of apigenin.

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Conflicts of Interest

Author declares that there are no conflicts of interest.

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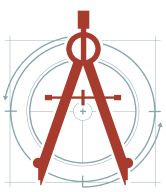

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