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RESEARCH ARTICLE

Synergistic Cytotoxicity of Cisplatin Combined with Curcumin and Green Tea Extract via Nanoliposomal Co-Delivery in Oral Squamous Cell Carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) remains one of the most prevalent malignancies worldwide, with cisplatin-based chemotherapy limited by systemic toxicity and drug resistance. The present study aimed to enhance cisplatin efficacy through nanoliposomal co-delivery with natural antioxidants curcumin (Cur) and green tea extract (epigallocatechin gallate, EGCG). **Methods:** Nanoliposomes were prepared via the thin-film hydration technique followed by sonication and extrusion, generating six formulations: cisplatin-loaded (L-Cis), curcumin-loaded (L-Cur), green tea extract-loaded (L-EGCG), cisplatin + curcumin (L-Cis/Cur), cisplatin + green tea extract (L-Cis/EGCG), and triple co-loaded nanoliposomes containing cisplatin, curcumin, and EGCG (L-Cis/Cur/EGCG) prepared according to a 1:6:6 molar ratio. **Results:** Physicochemical characterization revealed nanoscale particle size (212–279 nm), uniform distribution (PDI < 0.3), negative zeta potential (−19 to −23 mV), and high encapsulation efficiencies (69–84%). Scanning electron microscopy confirmed spherical morphology with smooth, homogeneous surfaces. The MTT assay demonstrated that co-loaded and triple-loaded liposomes exhibited significantly higher cytotoxicity than single-drug formulations ($p < 0.001$). While L-Cis reduced cell viability to approximately 50%, L-Cur and L-EGCG showed moderate effects (~65–70% viability). Co-formulations (L-Cis/Cur and L-Cis/EGCG) further decreased viability to 25–30%, and the triple co-loaded L-Cis/Cur/EGCG (1:6:6) formulation induced the strongest cytotoxic response, consistent with synergistic drug interaction. Live/Dead fluorescence imaging corroborated these findings, showing an elevated proportion of PI-positive apoptotic cells in the co-delivery groups, particularly in the triple-loaded system. **Conclusion:** Collectively, these results demonstrate that nanoliposomal co-encapsulation of cisplatin with curcumin and green tea extract enhances cytotoxic efficacy and apoptotic activity in OSCC cells compared to single-agent systems. The optimized L-Cis/Cur/EGCG (1:6:6) formulation exhibited the most favorable physicochemical properties and biological performance, highlighting its potential as a synergistic nanocarrier platform for oral cancer therapy with improved efficacy and reduced systemic toxicity.

Keywords: Oral squamous cell carcinoma (OSCC)- Cisplatin- Curcumin- Green tea extract (EGCG)- Nanoliposomes

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies of the oral cavity, accounting for over 90% of oral cancers worldwide [1]. Despite advances in surgical and chemotherapeutic strategies, the prognosis of OSCC remains poor, with high recurrence rates and significant treatment-related morbidity [1, 2]. Cisplatin, a platinum-based chemotherapeutic drug, is widely used as the first-line agent in OSCC treatment due to its strong cytotoxic activity [1, 3]. However, its clinical efficacy is often limited by severe systemic toxicity, drug resistance, and dose-dependent side effects, including nephrotoxicity and neurotoxicity [3, 4]. These limitations highlight the urgent need for novel therapeutic strategies that can enhance efficacy while reducing toxicity. Natural compounds with anticancer properties, such as curcumin (derived from Curcuma longa) and epigallocatechin-3-gallate (EGCG, the major catechin in green tea), have gained increasing attention as adjuvant agents in cancer therapy [5, 6]. Both compounds exhibit anti-proliferative, pro-apoptotic, and antioxidant properties, and several studies suggest their potential to sensitize tumor cells to chemotherapeutic agents, thereby reducing the required dose of cytotoxic drugs [7, 8]. However, their clinical application is hampered by poor solubility, instability in physiological conditions, and limited bioavailability [9, 10]. Nanotechnology-based drug delivery systems, particularly nanoliposomes, provide a promising platform to overcome these challenges. Liposomes are biocompatible, biodegradable vesicles capable of encapsulating both hydrophilic and hydrophobic drugs, protecting them from degradation, and facilitating controlled release at the tumor site [11]. Furthermore, co-encapsulation of chemotherapeutic drugs with natural compounds in liposomal carriers offers the potential for synergistic effects, enhanced tumor selectivity, and reduced systemic toxicity [12]. Given the growing physical and psychological challenges faced by modern societies, the importance of health and its crucial role in overall social well-being have become increasingly prominent [13-17]. Accordingly, there is a pressing need for experimental research at the laboratory level focusing on cancer and advanced drug delivery systems to develop more effective and safer therapeutic strategies [18-21]. In this study, we aimed to develop and characterize nanoliposomal formulations encapsulating cisplatin, curcumin, green tea extract, and their combinations. The physicochemical properties of the nanoliposomes were systematically analyzed by dynamic light scattering (DLS), zeta potential measurements, and scanning electron microscopy (SEM). Their cytotoxic and pro-apoptotic effects against OSCC cells were assessed using the MTT assay and Live/Dead fluorescence staining. We hypothesized that nanoliposomal co-delivery of cisplatin with natural extracts would enhance anticancer efficacy while reducing the effective dose of cisplatin, offering a more efficient and safer therapeutic strategy for OSCC.

Materials and Methods

Phosphatidylcholine (PC; Avanti Polar Lipids, USA), cholesterol (CHOL; Sigma-Aldrich, Germany), PEG-DSPE (Avanti Polar Lipids, USA), chloroform (Merck, Germany), methanol (Merck, Germany), cisplatin (Sigma-Aldrich, Germany), curcumin (Sigma-Aldrich, Germany), green tea extract standardized to EGCG (Cayman Chemical, USA), Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, USA), fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA), penicillin–streptomycin (Gibco, Thermo Fisher Scientific, USA), MTT reagent (Sigma-Aldrich, Germany), dimethyl sulfoxide (DMSO; Merck, Germany), acridine orange (AO; Sigma-Aldrich, Germany), propidium iodide (PI; Sigma-Aldrich, Germany), phosphate-buffered saline (PBS; Sigma-Aldrich, Germany), polycarbonate membranes (Whatman, UK).

Optimization of Cisplatin–Curcumin–Green Tea Extract Combination Ratio

The synergistic cytotoxic effect of cisplatin (Cis), curcumin (CUR), and epigallocatechin gallate (EGCG) on CAL-27 oral squamous carcinoma cells was evaluated using a cytotoxicity (MTT) assay. CAL-27 cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100 µL of complete medium and incubated for 24 hours at 37 °C in a 5% CO_2 incubator. After cell attachment, combinations of Cis, CUR, and EGCG at different molar ratios (1:3:3, 1:6:6, and 1:9:9) were added to the wells and incubated for 48 hours. Cell viability was then assessed by the MTT assay, and absorbance was measured at 570 nm using a microplate reader. For each cisplatin concentration, CUR and EGCG doses were scaled proportionally to maintain the specified Cis:CUR:EGCG ratios. To determine whether the drug interactions were synergistic, additive, or antagonistic, the median-effect (Chou–Talalay) method was applied. The results were analyzed by plotting the Combination Index (CI) against the fraction affected (Fa), where CI < 1 indicates synergy, CI = 1 denotes additivity, and CI > 1 indicates antagonism.

Preparation of Drug-Loaded Nanoliposomes

Nanoliposomes were prepared using the thin-film hydration method followed by probe sonication and extrusion to obtain nanosized unilamellar vesicles. A lipid mixture of phosphatidylcholine (PC), cholesterol (CHOL), and PEG-DSPE in a molar ratio of 65:30:5 was dissolved in chloroform–methanol (1:2, v/v), and the organic solvent was evaporated under reduced pressure at 40 °C using a rotary evaporator (Büchi, Switzerland) to form a thin lipid film. The film was further dried under vacuum for 2 h to ensure complete removal of residual solvent. For drug loading, the dried film was hydrated with phosphate-buffered saline (PBS, pH 7.4) containing the therapeutic agents according to the target formulation, resulting in six distinct liposomal systems: L-Cis (cisplatin-loaded), L-Cur (curcumin-loaded), L-EGCG (green tea extract-loaded, standardized to EGCG content), L-Cis/Cur

(cisplatin + curcumin), L-Cis/EGCG (cisplatin + green tea extract), and L-Cis/Cur/EGCG (triple co-loaded). For the co-loaded and triple-loaded formulations, drug concentrations were adjusted based on a 1:6:6 molar ratio of cisplatin:curcumin:EGCG to ensure synergistic balance in drug loading. Hydration was performed at 60 °C for 30 min with gentle agitation to form multilamellar vesicles, which were downsized by probe sonication (Qsonica, USA) for 5 min (30 s on/off cycles) and extruded through 200 nm polycarbonate membranes (Avanti Polar Lipids, USA) to obtain uniform nanoliposomes.

Characterization of Nanoliposomes

The physicochemical properties of the prepared nanoliposomes were characterized by multiple analytical techniques. Particle size distribution, polydispersity index (PDI), and zeta potential were measured using dynamic light scattering (DLS; Malvern Zetasizer Nano ZS90, UK) to assess colloidal stability. Morphological analysis of the vesicles was performed by scanning electron microscopy (SEM; JEOL JSM-IT300, Japan) to confirm the spherical shape and nanoscale structure. The encapsulation efficiency (EE%) and loading capacity (LC%) were determined for each drug. For curcumin and green tea extract (EGCG), quantification was performed using UV–Vis spectrophotometry (Shimadzu UV-1800, Japan) at their respective absorption maxima (425 nm for curcumin, 272 nm for EGCG). Cisplatin content was measured using high-performance liquid chromatography (HPLC; Agilent 1200 series, USA) equipped with a C18 reverse-phase column and UV detection at 254 nm. EE% and LC% were calculated using the following equations:

$$EE\ (\%) = (W\_Encapsulated / W\_Total) \times 100$$

$$LC\ (\%) = (W\_Encapsulated / (W\_Lipid + W\_Encapsulated) \times 100$$

where encapsulated W represents the weight of drug successfully entrapped in the nanoliposomes, total W is the initial drug weight, and lipid W is the weight of lipids used in the formulation.

In Vitro Drug Release Study

The in vitro drug release behavior of the nanoliposomal formulations was evaluated using the dialysis bag method under physiological conditions (pH 7.4). Each formulation was dispersed in phosphate-buffered saline (PBS, pH 7.4) containing 0.5% Tween-80 to maintain sink conditions for hydrophobic components. Two milliliters of each liposomal suspension were transferred into pre-soaked dialysis bags (MWCO 12–14 kDa; Sigma) and immersed in 50 mL of release medium maintained at 37 °C with continuous stirring (100 rpm). At predetermined intervals (0, 1, 2, 4, 8, 12, 24, 36, 48, and 52 h), 1 mL aliquots were withdrawn and replaced with an equal volume of fresh PBS to maintain constant volume. The released curcumin and EGCG concentrations were determined spectrophotometrically at 425 nm and 272 nm, respectively, while cisplatin release was quantified by HPLC at 254 nm. The cumulative percentage of released drug was calculated using the equation:

$$Cumulative\ Release\ (\%) = (W\_t / W\_0) \times 100$$

where Wt is the amount of drug released at time t, and W0 is the initial amount of drug encapsulated.

MTT Cell Viability Assay for CAL-27 Cells

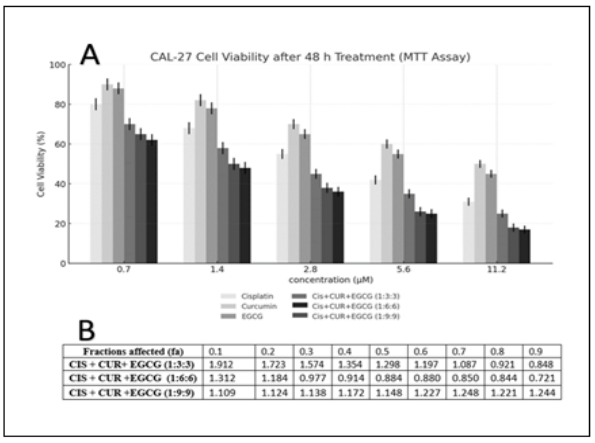
The cytotoxic effects of free and liposomal formulations containing cisplatin (Cis), curcumin (CUR), and green tea extract (EGCG) were evaluated on CAL-27 oral squamous carcinoma cells using the MTT assay. CAL-27 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 hours under standard conditions (37 °C, 5% CO_2, 95% humidity). After cell stabilization, cells were treated with various formulations, including single-agent liposomes, free drugs, and combination nanoliposomes containing Cis + Cur + EGCG (1:6:6). Treatments were applied for 48 hours. Following treatment, 20 µL of MTT reagent (5 mg/mL in PBS; Sigma) was added to each well and incubated for 4 hours at 37 °C to form formazan crystals. The supernatant was removed, and 150 µL of dimethyl sulfoxide (DMSO) was added to dissolve the crystals. Absorbance was recorded at 570 nm using a microplate reader (BioTek, USA). Cell viability (%) was calculated relative to untreated control cells.

Live/Dead Assay for Nanoliposomal Formulations

The cytotoxic and apoptotic effects of nanoliposome-encapsulated drug formulations were assessed in CAL-27 oral squamous carcinoma cells using a Live/Dead fluorescence assay based on acridine orange (AO; Sigma, A9231) and propidium iodide (PI; Sigma, P4864) staining. CAL-27 cells were seeded in 24-well plates at a density of 5×10^4 cells per well and incubated for 24 hours under standard culture conditions (37 °C, 5% CO_2) to allow cell attachment. Cells were subsequently treated with six nanoliposomal formulations: L-Cis, L-Cur, L-EGCG, L-Cis/Cur (cisplatin + curcumin), L-Cis/EGCG (cisplatin + green tea extract), and L-Cis/Cur/EGCG, the triple co-loaded formulation prepared according to a 1:6:6 molar ratio of cisplatin: curcumin: EGCG to achieve synergistic drug balance. After 48 hours of incubation, the cells were washed three times with PBS and stained with AO (5 µg/mL) and PI (10 µg/mL) for 15 minutes in the dark at room temperature. Viable cells emitted green fluorescence (AO uptake), whereas apoptotic or necrotic cells with compromised membranes displayed yellow-orange to red fluorescence (PI uptake). Fluorescence images were captured using an Olympus BX51 inverted fluorescence microscope equipped with appropriate filter sets at 20× magnification.

Statistical Analysis

Statistics were revised to reflect the actual analyses: one-way ANOVA with Tukey's post hoc test for MTT and two-way ANOVA with Tukey's post hoc test for release profiles; normality and variance assumptions



Fractions affected (fa)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
CIS + CUR+ EGCG (1:3:3)	1.912	1.723	1.574	1.354	1.298	1.197	1.087	0.921	0.848
CIS + CUR +EGCG (1:6:6)	1.312	1.184	0.977	0.914	0.884	0.880	0.850	0.844	0.721
CIS + CUR +EGCG (1:9:9)	1.109	1.124	1.138	1.172	1.148	1.227	1.248	1.221	1.244

Figure 1. Different Combination Ratios of Cisplatin, Curcumin, and Green Tea Extract Exhibit Varying Levels of Cell Viability (%) in CAL-27 Cells (A) Lower Bars Indicate Greater Growth Inhibition. The Combination Index (CI) plot (B) demonstrates the synergistic interaction among cisplatin, curcumin, and green tea extract, with the 1:6:6 ratio showing the greatest reduction in cell viability and the strongest synergistic effect

were checked, exact p values are reported, and data are presented as mean ± SD from at least three independent experiments with technical replicates.

Results

Optimization of Combination Ratio

As illustrated in Figure 1, synergistic effects were observed at higher levels of growth inhibition for the Cis:CUR:EGCG ratios of 1:3:3 and 1:6:6. However, the 1:9:9 ratio exhibited antagonistic behavior (CI > 1). Notably, the 1:6:6 formulation demonstrated a significant synergistic effect at an inhibition threshold above 30% (Fa = 0.3), which became even more pronounced at higher inhibition levels (Fa = 0.9). Therefore, the 1:6:6 ratio was identified as the optimal formulation and selected for subsequent studies involving the development of co-loaded nanoliposomes (Figure 1).

Morphological and Physicochemical Characterization

Scanning electron microscopy (SEM) revealed that all nanoliposomal formulations exhibited a predominantly spherical morphology with smooth and uniform surfaces, confirming successful vesicle formation (Figure 2). The particle size increased progressively with drug incorporation, indicating the effect of encapsulated agents on lipid packing and vesicle curvature. Among the single-drug formulations, L-Cis, L-Cur, and L-EGCG displayed mean diameters of 212.3 ± 3.8 nm, 229.5 ± 4.5 nm, and 238.2 ± 5.0 nm, respectively. The co-loaded systems, L-Cis/Cur and L-Cis/EGCG, showed slightly larger sizes (258.7 ± 4.9 nm and 266.4 ± 5.1 nm), reflecting the combined incorporation of multiple bioactives within the lipid bilayer. The triple co-loaded formulation, L-Cis/Cur/EGCG (1:6:6), exhibited the largest particle size (278.9 ± 5.3 nm), consistent with its higher drug content and more complex internal structure.All formulations demonstrated narrow size distributions (PDI < 0.3), indicating homogeneity and effective size control after sonication and extrusion. The zeta potential values ranged from –19.6 mV to –23.1 mV, confirming the formation of stable, negatively charged vesicles due to the presence of phosphatidylcholine and PEG-DSPE. Drug encapsulation efficiency (EE%) varied between 68.7% and 83.5%, while the loading capacity (LC%) ranged from 10.4% to 14.1%. The highest EE% and LC% were observed in the L-Cis/Cur/EGCG (1:6:6) formulation, indicating favorable drug–lipid interactions and enhanced retention within the vesicle matrix. Overall, these findings confirm the successful fabrication of nanosized, monodisperse, and stable liposomal systems capable of efficiently co-encapsulating cisplatin, curcumin, and green tea extract for potential synergistic anticancer therapy (Figure 2 and Table 1).

In Vitro Drug Release Profiles

The cumulative release curves of all nanoliposomal formulations are presented in Figure 3. The blank liposomes exhibited negligible release (< 5%), confirming the stability of the lipid matrix and the absence of encapsulated drug. In contrast, drug-loaded systems displayed clearly distinct kinetic profiles, indicating differences in diffusion mechanisms and lipid–drug interactions. Single-drug liposomes showed faster release rates, with L-Cis, L-Cur, and L-EGCG reaching approximately 90%, 88%, and 83% cumulative release, respectively, after 52 hours. Dual co-loaded systems (L-Cis/Cur and L-Cis/EGCG) exhibited moderately sustained release (≈ 70–75%), while the triple co-loaded formulation (L-Cis/Cur/EGCG) demonstrated the most controlled and prolonged release, achieving only about 55% total release within 52 hours.These findings confirm that co-encapsulation of cisplatin with natural antioxidants (curcumin and EGCG) not only modifies the lipid–drug interactions but also results in more controlled release

Table 1. Physicochemical Characteristics of Drug-loaded Liposomal Formulations

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)	EE%	LC%
L-Cis	212.3 ± 3.8	0.23 ± 0.01	–22.6 ± 0.7	79.1 ± 1.4	12.3 ± 0.5
L-Cur	229.5 ± 4.5	0.24 ± 0.07	–23.1 ± 0.6	73.4 ± 1.6	11.6 ± 0.6
L-EGCG	238.2 ± 5.0	0.25 ± 0.02	–21.9 ± 0.9	68.7 ± 1.8	10.4 ± 0.5
L-Cis/Cur	258.7 ± 4.9	0.26 ± 0.05	–20.8 ± 0.8	77.8 ± 1.5	13.0 ± 0.7
L-Cis/EGCG	266.4 ± 5.1	0.25 ± 0.06	–20.3 ± 0.7	81.2 ± 1.7	13.5 ± 0.6
L-Cis/Cur/EGCG (1:6:6)	278.9 ± 5.3	0.27 ± 0.02	–19.6 ± 0.8	83.5 ± 1.8	14.1 ± 0.8

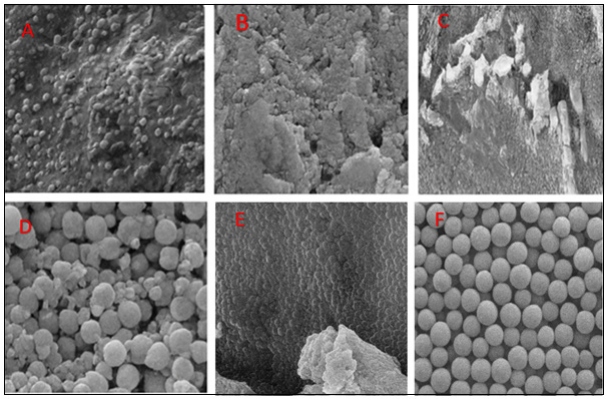


Figure 2. Representative Scanning Electron Microscopy (SEM) Images of Nanoliposomal Formulations: (A) cisplatin-loaded liposomes (L-Cis), (B) curcumin-loaded liposomes (L-Cur), (C) green tea extract-loaded liposomes (L-EGCG), (D) cisplatin + curcumin co-loaded liposomes (L-Cis/Cur), (E) cisplatin + green tea extract co-loaded liposomes (L-Cis/EGCG), and (F) triple co-loaded liposomes containing cisplatin, curcumin, and green tea extract (L-Cis/Cur/EGCG, 1:6:6).

kinetics, providing a mechanistic basis for enhanced therapeutic synergy in subsequent cytotoxicity studies (Figure 3).

Cytotoxicity of Drug-Loaded Nanoliposomes in CAL-27 Cells

The MTT results demonstrated that blank liposomes exhibited negligible cytotoxicity, maintaining nearly 100% cell viability. Free cisplatin induced a substantial reduction in viability (~45%), while free curcumin and free EGCG displayed moderate effects (~65–70% viability). Liposomal single-agent formulations further enhanced cytotoxicity compared with their free counterparts, confirming improved intracellular delivery. Notably, the co-loaded nanoliposome containing Cis + Cur + EGCG (1:6:6) exhibited the most potent cytotoxic effect, reducing cell viability to below 25% after 48 hours ($p < 0.001$). This combined formulation demonstrated a clear synergistic interaction, significantly enhancing CAL-27 cell death compared with individual or dual-agent liposomes ($p < 0.05$). These findings confirm that co-delivery of cisplatin with natural antioxidants (CUR and EGCG) within a liposomal carrier greatly enhances cytotoxic efficacy while potentially minimizing the required cisplatin dose. The observed synergy supports the selection of this optimized combination (1:6:6) for subsequent nanoliposomal development and characterization (Figure 4).

Live/Dead Assay Outcomes

Representative fluorescence microscopy images of CAL-27 cells treated with different nanoliposomal formulations are presented in Figure 4. The single-drug liposomes (L-Cis, L-Cur, and L-EGCG) displayed distinct cytotoxicity levels consistent with their individual potency. L-Cis caused substantial cell death, with approximately 50% of cells exhibiting red/orange propidium iodide (PI) fluorescence, indicative of apoptosis and necrosis. In contrast, L-Cur and L-EGCG demonstrated moderate cytotoxicity, with about 30–35% loss in viability relative to untreated controls, confirming their mild but measurable pro-apoptotic effects. The co-loaded systems, L-Cis/Cur and L-Cis/EGCG, produced enhanced cytotoxic responses, reducing viability to roughly 25–30%, with dense PI-positive staining across the cell monolayer. Notably, the triple co-loaded formulation L-Cis/Cur/EGCG (1:6:6) (Figure 5F) exhibited the most pronounced cytotoxic and pro-apoptotic activity, showing the lowest number of viable (green-fluorescent) cells and the highest intensity of red/orange fluorescence. This observation confirms that the simultaneous delivery of cisplatin, curcumin, and green tea extract produces a synergistic enhancement of cytotoxicity in CAL-27 oral cancer cells. These findings are in strong agreement with the MTT assay results, further validating the superior therapeutic potential of the L-Cis/Cur/EGCG formulation ($p < 0.001$).

Discussion

In this study, six nanoliposomal formulations were successfully developed to evaluate the synergistic cytotoxic potential of cisplatin combined with natural bioactives curcumin and green tea extract (EGCG) against oral squamous cell carcinoma (CAL-27) cells. The results revealed that co-encapsulation of cisplatin with curcumin (L-Cis/Cur), cisplatin with EGCG (L-Cis/EGCG), and particularly the triple co-loaded formulation (L-Cis/Cur/EGCG, 1:6:6) markedly enhanced cytotoxicity and apoptotic activity compared with single-agent formulations (L-Cis, L-Cur, and L-EGCG). Among these, the L-Cis/Cur/EGCG (1:6:6) system demonstrated the most potent effect, reducing CAL-27 cell viability to

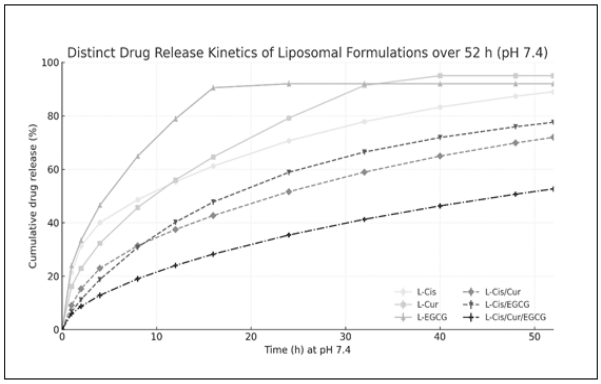


Figure 3. In Vitro Cumulative Drug Release Profiles of Six Liposomal Formulations at Physiological pH (7.4) Over 52 Hours. Distinct kinetic patterns were observed among the formulations, indicating differences in diffusion and membrane permeability. Single-drug liposomes (L-Cis, L-Cur, and L-EGCG) exhibited a biphasic release behavior with an initial burst phase followed by a diffusion-controlled phase. Dual co-loaded systems (L-Cis/Cur and L-Cis/EGCG) showed moderated and more sustained release profiles, while the triple co-loaded formulation (L-Cis/Cur/EGCG) demonstrated the slowest and most controlled drug release, reaching approximately 55% cumulative release after 52 hours.

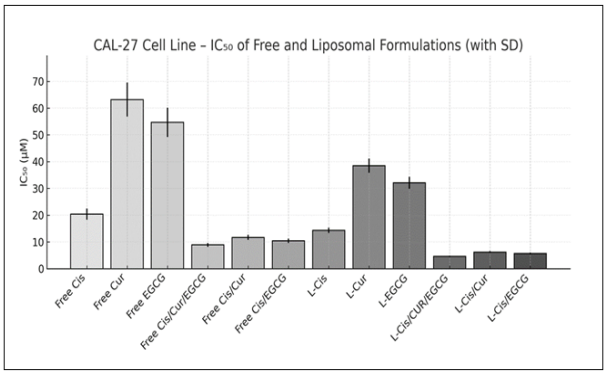


Figure 4. MTT Assay Results Showing the Cytotoxic Effects of Free Drugs and Liposomal Formulations on CAL-27 Oral Squamous Carcinoma Cells after 48 Hours of Treatment. Blank liposomes maintained nearly complete cell viability, confirming biocompatibility. Free cisplatin, curcumin, and green tea extract (EGCG) displayed moderate cytotoxicity, while their liposomal counterparts demonstrated enhanced efficacy. The co-loaded liposomal formulation containing CIS + CUR + EGCG (1:6:6) exhibited the greatest reduction in cell viability (<25%), indicating a strong synergistic effect and improved intracellular delivery compared with single or dual-agent formulations.

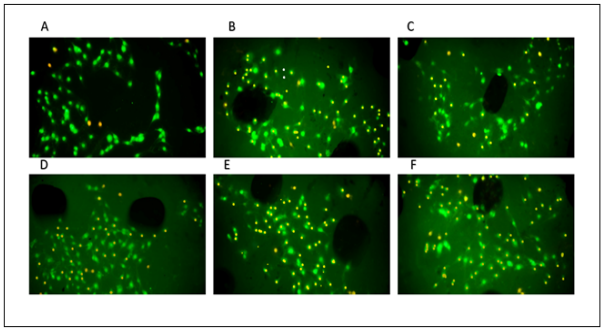


Figure 5. Representative Live/Dead Fluorescence Microscopy Images of CAL-27 Cells after 48-hour Treatment with Nanoliposomal Formulations: (A)) curcumin-loaded liposomes (L-Cur) (B) cisplatin-loaded liposomes (L-Cis, (C) green tea extract-loaded liposomes (L-EGCG), (D) cisplatin + curcumin co-loaded liposomes (L-Cis/Cur), (E) cisplatin + green tea extract co-loaded liposomes (L-Cis/EGCG), and (F) triple co-loaded liposomes containing cisplatin, curcumin, and green tea extract (L-Cis/Cur/EGCG, 1:6:6). Green fluorescence (AO) represents viable cells, whereas red/orange fluorescence (PI) indicates apoptotic or necrotic cells. The co-loaded and triple-loaded formulations exhibited markedly higher cytotoxicity, as evidenced by the increased proportion of red/orange fluorescence, which is consistent with the MTT assay results

approximately 20–25%, consistent with strong synergistic interactions among the three compounds. These findings are consistent with previous studies reporting enhanced anticancer efficacy through co-delivery of cisplatin with natural antioxidants. Cheng et al. (2018) demonstrated that curcumin–cisplatin co-loaded liposomes exerted synergistic cytotoxic effects in HepG2 hepatocellular carcinoma cells while reducing systemic toxicity [22]. Similarly, Hu et al. (2015) showed that EGCG sensitized colorectal cancer cells to cisplatin via autophagy-mediated apoptosis, underscoring the potential of natural compounds to modulate cellular stress pathways and improve chemotherapy outcomes [23]. In the context of oral cancer, Saeidi et al. (2024) reported that cisplatin–curcumin nanoliposomes significantly enhanced cytotoxicity in CAL-27 cells and exhibited sustained release behavior, supporting the rationale for combination nanocarrier therapy [24]. From a physicochemical standpoint, all six formulations exhibited nanoscale particle size (212–279 nm), negative zeta potential, and high encapsulation efficiency (> 68%), confirming the stability and suitability of these vesicles for drug delivery [25]. The gradual increase in particle size from single- to multi-drug systems suggested successful co-encapsulation and increased lipid bilayer packing complexity [26]. Compared with prior formulations such as Cheng's (~294 nm vesicles), the smaller and more uniform liposomes developed in this study may offer improved tissue penetration and pharmacokinetic behavior [22]. Scanning electron microscopy (SEM) analysis confirmed the successful preparation of spherical, nanosized liposomes across all formulations, with smooth surfaces and uniform morphology. The absence of aggregation and structural irregularities suggested effective encapsulation and strong lipid bilayer integrity [27]. Notably, the triple co-loaded formulation (L-Cis/Cur/EGCG, 1:6:6) exhibited the most compact and homogeneous surface among all samples, which may contribute to its enhanced colloidal stability and controlled drug release behavior [28]. The in vitro release profiles revealed distinct kinetic patterns for each formulation at physiological pH 7.4. Single-drug liposomes (L-Cis, L-Cur, L-EGCG) demonstrated moderate sustained release, whereas co-loaded formulations displayed slower and more regulated release patterns, indicating strong drug–lipid interactions within the bilayer. Among them, the L-Cis/Cur/EGCG (1:6:6) system exhibited the slowest cumulative release (~54% at 52 h), supporting the notion that multiple hydrophobic and hydrophilic drug interactions contribute to matrix densification and diffusion-controlled kinetics. These results are consistent with previous reports describing reduced burst release and prolonged diffusion for multi-drug nanocarriers [29-31]. The MTT cytotoxicity assay further demonstrated that the liposomal encapsulation of cisplatin in combination with natural antioxidants (curcumin or EGCG) significantly enhanced the anticancer efficacy against CAL-27 cells. Free drug formulations showed relatively high IC_{50} values (20–60 μM), whereas nanoliposomal systems markedly reduced these values to below 10 μM. The L-Cis/Cur/EGCG (1:6:6) formulation achieved the lowest IC_{50} (≈ 5 μM), reflecting a synergistic enhancement of cytotoxicity through co-encapsulation. These findings align with the synergistic effects previously reported for cisplatin–curcumin [22] and cisplatin–EGCG combinations [23]. Fluorescence Live/Dead imaging visually corroborated the MTT results. A predominance of green fluorescence was observed in untreated controls, confirming healthy viable cells, while red/orange fluorescence increased markedly following treatment with drug-loaded liposomes. The L-Cis/Cur/EGCG (1:6:6)

group exhibited the most intense PI-positive staining, indicating extensive apoptosis and necrosis. This visual evidence supports the conclusion that triple co-delivery significantly enhances apoptotic cell death compared to single-drug or dual-drug formulations [32]. Overall, our findings demonstrate that nanoliposomal co-delivery of cisplatin with curcumin and EGCG particularly the optimized L-Cis/Cur/EGCG (1:6:6) formulation represents a promising therapeutic approach for oral cancer. This strategy offers synergistic anticancer activity, improved apoptosis induction, and potential reduction in cisplatin-associated toxicity, positioning it as a rational design for next-generation combination chemotherapy systems.

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Statements and Declarations

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval

This study did not involve any original data collection or human subjects, and therefore, ethical approval was not required.

Consent

All authors have provided consent for publication.

Originality Declaration for Figures

All figures included in this manuscript are original and have been created by the authors specifically for the purposes of this study. No previously published or copyrighted images have been used. The authors confirm that all graphical elements, illustrations, and visual materials were generated from the data obtained in the course of this research or designed uniquely for this manuscript.

References

1. Wang H, Ma Y. β-Elemene alleviates cisplatin resistance in oral squamous cell carcinoma cell via inhibiting JAK2/STAT3 pathway in vitro and in vivo. Cancer Cell International. 2022 07 31;22(1):244. https://doi.org/10.1186/s12935-022-02650-7
2. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncology. 2009;45(4-5):309-316. https://doi.org/10.1016/j.oraloncology.2008.06.002
3. Galluzzi L, Vitale I, Michels J, Brenner C, Szabadkai G, Harel-Bellan A, Castedo M, Kroemer G. Systems biology of cisplatin resistance: past, present and future. Cell Death & Disease. 2014 05 29;5(5):e1257. https://doi.org/10.1038/cddis.2013.428
4. Santos NAG, Carvalho Rodrigues MA, Martins NM, Santos AC. Cisplatin-induced nephrotoxicity and targets of nephroprotection: an update. Archives of Toxicology. 2012 08;86(8):1233-1250. https://doi.org/10.1007/s00204-012-0821-7
5. Chung SS, Vadgama JV. Curcumin and epigallocatechin gallate inhibit the cancer stem cell phenotype via down-regulation of STAT3-NFκB signaling. Anticancer Research. 2015 01;35(1):39-46. https://doi.org/kkkk
6. Sohn S, Priya A, Balasubramaniam B, Muthuramalingam P, Sivasankar C, Selvaraj A, Valliammai A, Jothi R, Pandian S. Biomedical Applications and Bioavailability of Curcumin-An Updated Overview. Pharmaceutics. 2021 Dec 07;13(12):2102. https://doi.org/10.3390/pharmaceutics13122102
7. Eom D, Lee JH, Kim Y, Hwang GS, Kim S, Kwak JH, Cheon GJ, et al. Synergistic effect of curcumin on epigallocatechin gallate-induced anticancer action in PC3 prostate cancer cells. BMB reports. 2015 08;48(8):461-466. https://doi.org/10.5483/bmbrep.2015.48.8.216
8. Cui F, Han S, Wang J, McClements DJ, Liu X, Liu F. Co-delivery of curcumin and epigallocatechin gallate in W/O/W emulsions stabilized by protein fibril-cellulose complexes. Colloids and Surfaces. B, Biointerfaces. 2023 02;222:113072. https://doi.org/10.1016/j.colsurfb.2022.113072
9. Pandit AP, Joshi SR, Dalal PS, Patole VC. Curcumin as a permeability enhancer enhanced the antihyperlipidemic activity of dietary green tea extract. BMC complementary and alternative medicine. 2019 06 13;19(1):129. https://doi.org/10.1186/s12906-019-2545-1
10. Chen Y, Jia D, Wang Q, Sun Y, Rao Z, Lei X, Zhao J, et al. Promotion of the anticancer activity of curcumin based on a metal-polyphenol networks delivery system. International Journal of Pharmaceutics. 2021 06 01;602:120650. https://doi.org/10.1016/j.ijpharm.2021.120650
11. Liu P, Chen G, Zhang J. A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. Molecules (Basel, Switzerland). 2022 02 17;27(4):1372. https://doi.org/10.3390/molecules27041372
12. Gabizon A, Ohana P, Amitay Y, Gorin J, Tzemach D, Mak L, Shmeeda H. Liposome co-encapsulation of anti-cancer agents for pharmacological optimization of nanomedicine-based combination chemotherapy. Cancer Drug Resistance (Alhambra, Calif.). 2021;4(2):463-484. https://doi.org/10.20517/cdr.2020.87
13. Nawaser K, Jafarkhani F, Khamoushi S, Yazdi A, Mohsenifard H, Gharleghi B. The Dark Side of Digitalization: A Visual Journey of Research through Digital Game Addiction and Mental Health. IEEE Engineering Management Review. 2024;:1-27. https://doi.org/10.1109/EMR.2024.3462740
14. Mohammadabadi SMS, Seyedkhamoushi F, Mostafavi M, Peikani MB. Examination of AI's Role in Diagnosis, Treatment, and Patient Care. Transforming Gender-Based Healthcare with AI and Machine Learning. 2024 Oct 03;:221-238. https://doi.org/nnnnn
15. Basirat S, Raoufi S, Bazmandeh D, Khamoushi S, Entezami M. Ranking of AI-Based Criteria in Health Tourism Using Fuzzy SWARA Method. Computer and Decision Making: An International Journal. 2025 03 30;2:530-545. https://doi.org/10.59543/comdem.v2i.13795
16. Seifi N, Ghoodjani E, Majd SS, Maleki A, Khamoushi S. Evaluation and prioritization of artificial intelligence integrated block chain factors in healthcare supply chain: A hybrid Decision Making Approach. Computer and Decision Making: An International Journal. 2025 01 05;2:374-405. https://doi.org/10.59543/comdem.v2i.11029
17. Mehrabi Jorshary K, Sassani M, Seyed Khamoushi FS, Raoufi S, Khamoushi S. Ranking of AI-Driven Strategies for Optimizing the Health Tourism Supply Chain Using Stratified BWM. Knowledge and Decision Systems

with Applications. 2025 09 18;1:295-315. https://doi.org/10.59543/kadsa.v1i.15051
18. Jafarkhani S, Amiri E, Moazzeni S, Zohoorian-Abootorabi T, Eftekhary M, Aminnezhad S, Khakbiz M. Exploring the effects of micro-nano surface topography on MG63 osteoblast-like cell responses: An in vitro study. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2023 Oct;675:131872. https://doi.org/10.1016/j.colsurfa.2023.131872
19. Khakbiz M, Chagami M, Sheibani S, Amiri E, Moazzeni S, Shakibania S, Hou Y, Lee K. Enhancement of corrosion, biocompatibility and drug delivery properties of nitinol implants surface by Al-Zn-LDH nanohybrids. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2025 01;704:135524. https://doi.org/10.1016/j.colsurfa.2024.135524
20. Bardajee GR, Shafiei N, Rouhi M, Mahmoodian H. Inverse Miniemulsion Polymerization of Dual-Responsive Single-Network Alg-PDMAEMA Nanogels Colon-Targeted DOX Delivery. Journal of Cluster Science. 2025 07 11;36(4):149. https://doi.org/10.1007/s10876-025-02865-4
21. Golkarieh A, Razmara P, Lagzian A, Dolatabadi A, Mousavirad SJ. Semi-supervised GAN with hybrid regularization and evolutionary hyperparameter tuning for accurate melanoma detection. Scientific Reports. 2025 08 30;15(1):31977. https://doi.org/10.1038/s41598-025-17756-x
22. Cheng Y, Zhao P, Wu S, Yang T, Chen Y, Zhang X, He C, et al. Cisplatin and curcumin co-loaded nano-liposomes for the treatment of hepatocellular carcinoma. International Journal of Pharmaceutics. 2018 07 10;545(1-2):261-273. https://doi.org/10.1016/j.ijpharm.2018.05.007
23. Hu F, Wei F, Wang Y, Wu B, Fang Y, Xiong B. EGCG synergizes the therapeutic effect of cisplatin and oxaliplatin through autophagic pathway in human colorectal cancer cells. Journal of Pharmacological Sciences. 2015 05;128(1):27-34. https://doi.org/10.1016/j.jphs.2015.04.003
24. Saeidi N, Ansarikojouri M, Mardani M, Rezazadeh R, Goodarzi MK, Amiri F. Enhancing the Cytotoxic Effects of Carboplatin and Cisplatin on Liposomes in Oral Cancer Cells with Curcumin. Asian Pacific Journal of Cancer Biology. 2024 Nov 26;9(4):579-587. https://doi.org/10.31557/apjcb.2024.9.4.579-587
25. Bozzuto G, Molinari A. Liposomes as nanomedical devices. International Journal of Nanomedicine. 2015;10:975-999. https://doi.org/10.2147/IJN.S68861
26. Ong SGM, Ming LC, Lee KS, Yuen KH. Influence of the Encapsulation Efficiency and Size of Liposome on the Oral Bioavailability of Griseofulvin-Loaded Liposomes. Pharmaceutics. 2016 08 26;8(3):25. https://doi.org/10.3390/pharmaceutics8030025
27. Zhang W, Taheri-Ledari R, Ganjali F, Mirmohammadi SS, Qazi FS, Saeidirad M, KashtiAray A, et al. Effects of morphology and size of nanoscale drug carriers on cellular uptake and internalization process: a review. RSC advances. 2022 Dec 19;13(1):80-114. https://doi.org/10.1039/d2ra06888e
28. Lombardo D, Kiselev MA. Methods of Liposomes Preparation: Formation and Control Factors of Versatile Nanocarriers for Biomedical and Nanomedicine Application. Pharmaceutics. 2022 02 28;14(3):543. https://doi.org/10.3390/pharmaceutics14030543
29. Lee JH, Yeo Y. Controlled Drug Release from Pharmaceutical Nanocarriers. Chemical Engineering Science. 2015 03 24;125:75-84. https://doi.org/10.1016/j.ces.2014.08.046
30. Zhdanov VP. Release of molecules from nanocarriers. Physical chemistry chemical physics: PCCP. 2023 Nov 01;25(42):28955-28964. https://doi.org/10.1039/d3cp01855e
31. Kozik V, Pentak D, Październik M, Zięba A, Bąk A. From Design to Study of Liposome-Driven Drug Release Part 1: Impact of Temperature and pH on Environment. International Journal of Molecular Sciences. 2023 07 20;24(14):11686. https://doi.org/10.3390/ijms241411686
32. Filipczak N, Jaromin A, Piwoni A, Mahmud M, Sarisozen C, Torchilin V, Gubernator J. A Triple Co-Delivery Liposomal Carrier That Enhances Apoptosis via an Intrinsic Pathway in Melanoma Cells. Cancers. 2019 Dec 09;11(12):1982. https://doi.org/10.3390/cancers11121982