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Enhancing the In Vitro Therapeutic Efficiency of Epigallocatechin-3-Gallate in Esophageal Carcinoma Using Bovine Milk Exosomes as a Nanocarrier

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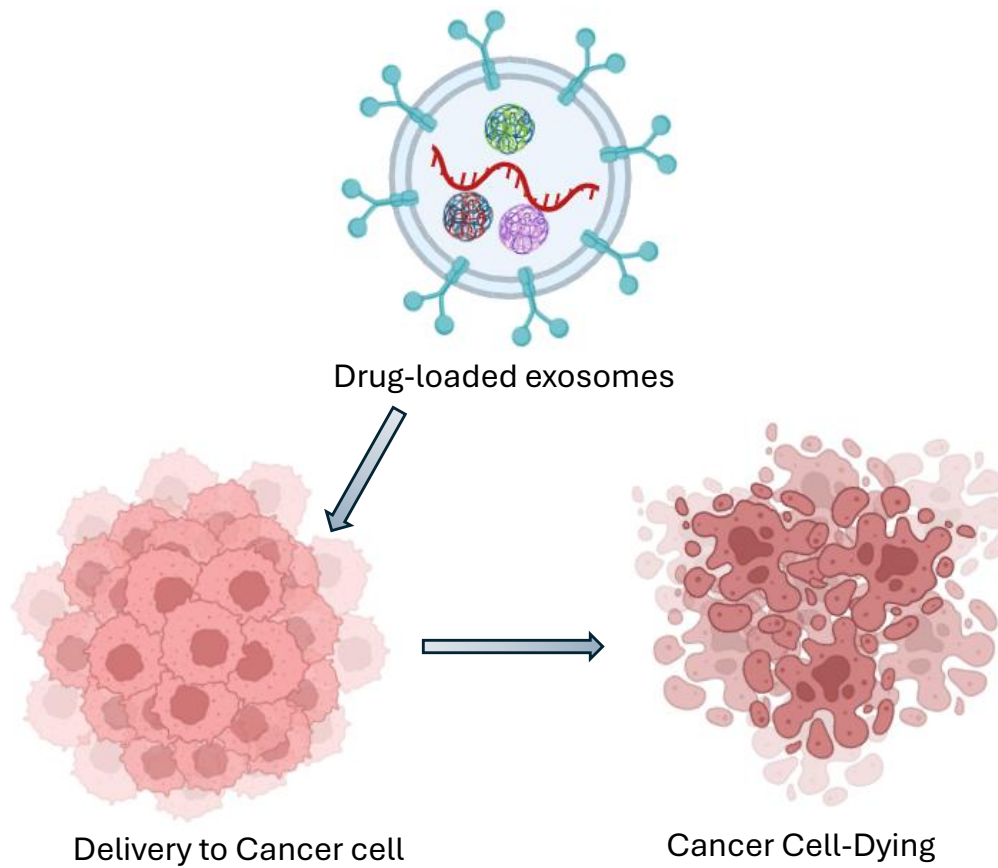
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Graphical Abstract



Abstract:

Esophageal adenocarcinoma (EAC) is an aggressive malignancy characterized by severe therapeutic resistance and a dismal prognosis. Although the green tea polyphenol epigallocatechin-3-gallate (EGCG) possesses potent anti-cancer properties, its clinical translation is restricted by poor stability and low bioavailability. To overcome these bottlenecks, we engineered a biocompatible nanoplatform utilizing bovine milk exosomes to encapsulate EGCG (Exo-EGCG). The resulting formulations exhibited favorable physicochemical attributes, including an average hydrodynamic diameter of 130.6 ± 3.6 nm, excellent colloidal stability, high encapsulation efficiency ($78.5 \pm 5.6\%$), and a sustained, pH-responsive biphasic release profile accelerated at pH 5.5.

In vitro screens against human EAC lines (SKGT-4 and FLO-1) demonstrated that exosomal encapsulation drastically enhanced the anti-proliferative and anti-clonogenic performance of EGCG. MTT profiling revealed time- and dose-dependent reductions in IC_{50} values, driven by enhanced exosomes mediated endocytosis. Furthermore, Exo-EGCG triggered a catastrophic intracellular oxidative burst, characterized by a significant ($p < 0.05$) upsurge in global cytoplasmic reactive oxygen species (ROS) and localized mitochondrial superoxide overproduction, effectively crippling the mitochondrial network. Furthermore, sub-toxic concentrations ($15 \mu\text{M}$) of Exo-EGCG markedly suppressed lateral and transwell cell migration by attenuating essential pro-metastatic drivers. FLO-1 cells exhibited more rapid baseline migration and higher susceptibility to drug-induced oxidative collapse than the heterogeneous SKGT-4 line. Collectively, these findings demonstrate that bovine milk exosome encapsulation successfully bypasses the delivery barriers of EGCG, establishing a scalable and potent nanomedicine platform for refractory esophageal adenocarcinoma.

Keyword: Epigallocatechin-3-gallate; exosomes; bovine milk exosomes; Esophageal adenocarcinoma

Introduction:

EAC remains largely resistant to current standard-of-care treatment options, with approximately 70% of cases exhibiting refractory disease and recently approved immunotherapies providing only a marginal extension of overall survival by a few months [1]. As a highly aggressive upper gastrointestinal malignancy, EAC carries an exceptionally poor prognosis; because most patients are diagnosed at an advanced clinical stage, the five-year survival rate is only approximately 20%. The public health impact of this malignancy is particularly evident in the United States[2, 3], where EAC accounts for the majority of the estimated 22,070 new esophageal cancer cases diagnosed annually—a figure that reflects a steady increase in incidence across Western nations over the last three decades[4, 5]. Consequently, there is an urgent requirement for the development of novel therapeutic strategies that demonstrate both high anti-tumor activity and a favourable, low-toxicity profile [6].

Extensive epidemiological and clinical data indicate that daily green tea consumption significantly promotes human health, particularly through its chemopreventive and cardiovascular benefits. Globally, numerous studies have highlighted the site-specific efficacy of green tea against various malignancies [7, 8]. In Asian cohorts, a Japanese study demonstrated that high-volume green tea intake correlates with a reduced risk of gastric cancer [9], while research in China suggested a protective effect against esophageal cancer

development[10]. Similarly, clinical investigations in Western populations support these findings: a trial in the United States found that green tea extract has therapeutic potential against prostate cancer progression[11], and European cohort data indicated a modest inverse correlation with breast cancer incidence[12]. Beyond oncology, long-term longitudinal studies have demonstrated that regular green tea consumption is associated with a 16% to 26% reduction in cardiovascular-related mortality [13]. Furthermore, meta-analyses of randomized controlled trials (RCTs) confirm that regular intake improves glycemic control by significantly lowering fasting blood glucose, circulating insulin levels, and HbA1c [14]. These multi-targeted pharmacological properties are fundamentally attributed to the plant's primary bioactive constituents, specifically the potent polyphenol epigallocatechin-3-gallate (EGCG) and the amino acid L-theanine [15].

Despite the well-documented pharmacological advantages of EGCG, including its potent chemopreventive and anticancer properties, its clinical translation is severely hindered by unfavourable pharmacokinetic attributes. The primary challenges restricting the therapeutic utility of EGCG in clinical settings are its poor aqueous stability and low oral bioavailability [16, 17]. Following administration, these limitations lead to rapid metabolic degradation and systemic clearance, ultimately compromising its therapeutic efficacy at target tumor sites. Consequently, the development of an advanced delivery vehicle is indispensable to optimizing the pharmacokinetic profile of EGCG, enhancing its stability, and maximizing its therapeutic potential [18].

To overcome these pharmacokinetic limitations, various nano-based systems have been engineered, including gold nanoparticles, polymeric nanoparticles, liposomal formulations, and folic acid-targeted nanocarriers [18-20]. However, each platform faces distinct clinical bottlenecks. Specifically, gold nanoparticles are limited by poor biodegradability and long-term systemic toxicity, while polymeric nanoparticles frequently exhibit low drug-loading capacity and premature payload release. Furthermore, conventional liposomes undergo rapid elimination by the reticuloendothelial system (RES), and active targeting via folic acid functionalization is often compromised by receptor saturation in vivo [21]. Consequently, the efficient therapeutic delivery of EGCG still requires a novel, optimized vehicle capable of surmounting these collective delivery challenges [22]. By bridging the gap between artificial nanocarriers and biological compatibility, exosome-based delivery systems offer a comprehensive solution to the pharmacokinetic hurdles of EGCG, paving the way for more effective cancer therapeutics.

Among biological nanocarriers, bovine milk-derived exosomes have emerged as highly promising drug delivery vehicles [23]. Naturally functioning as extracellular vesicles that traffic nucleic acids and proteins for intercellular communication, these endogenous nanoparticles offer exceptional biocompatibility, negligible toxicity, cost-effectiveness, and a prolonged systemic half-life [24]. Because their unique amphiphilic architecture enables the encapsulation of both hydrophilic and hydrophobic compounds, they are increasingly utilized to deliver diverse small-molecule chemotherapeutics [25]. Consequently, utilizing bovine milk exosomes presents an ideal strategy to optimize the delivery of EGCG.

To the best of our knowledge, no study has yet evaluated the therapeutic deployment of EGCG encapsulated within bovine milk exosomes for the treatment of esophageal adenocarcinoma (EAC). Therefore, this study investigates the *in vitro* anticancer efficacy of exosome-loaded EGCG (Exo-EGCG) against established EAC cell lines. We hypothesize that this biologically derived delivery strategy will achieve enhanced cellular internalization, superior cytotoxicity

against malignant cells, and diminished off-target effects compared to free EGCG, ultimately establishing a scalable therapeutic platform with high translational potential.

Materials and methods

Epigallocatechin-3-gallate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), crystal violet, 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), Coumarine-6, were purchased from Sigma-Aldrich. BCA kit, DMEM medium, F-12 medium, and penicillin-streptomycin solution were purchased from Thermofisher. Primary antibodies for Alix, TSG101, and CD63, and secondary antibodies were purchased from CST. Mitochondrial Superoxide Assay Kit, Cellular ROS Assay Kit (Deep Red). All other chemicals, unless notified otherwise, were used from the highest-grade quality.

Isolation of exosomes

Fresh whole milk was purchased from the nearest superstore and immediately processed for exosome isolation. First step: whole milk was centrifuged for 30 minutes at 13×10^3 RCF to remove the fatty layer; the supernatant was carefully collected, then ultracentrifuged at 1×10^5 RCF for 60 minutes at 4 °C. In the final step to collect exosomes, supernatant was taken from the above step, then ultracentrifuged again at 1.3×10^6 for 90 minutes at 4 °C. The exosomes pellets were collected from the bottom of the centrifuge tube, then homogenized with a Potter Elvehjem homogenizer to make monodispersed particles, followed by protein estimation with a BCA kit and stored at -80 until use [26].

Western blotting for confirmation

To confirm the purity of the exosomes, we performed Western blot analysis for exosome markers such as Alix, TSG101, and CD81. Additionally, to confirm purity, we performed Western blotting for markers of multivesicular bodies (MVBs), including β 1 integrin, P-selectin, and CD40 [27]. Briefly, exosomes were lysed with RIPA buffer, and the protein concentration was estimated using a BCA assay. An equal amount of protein (30 μ g) from different batches was then mixed with Laemmli buffer, after which the gel was run. This was followed by blocking with 5% BSA, incubation with rabbit primary antibodies, and subsequent incubation with anti-rabbit secondary antibodies. Finally, the blots were treated with an ECL solution and imaged using a ChemiDoc (Bio-Rad) imaging system.

Drug loading and encapsulation efficiency

Epigallocatechin-3-gallate-loaded exosomes (Exo-EGCG) were prepared via the sonication method as previously mentioned protocol [28]. Briefly, EGCG was dissolved in phosphate-buffered saline (PBS) and then incubated with exosomes (1:9) 1 mg of drug and 9 mg of exosomes) for 5 minutes, followed by sonication. We processed the sample for three minutes using six cycles of 30-second bursts and 30-second rests, operating a 0.25-inch probe sonic sonicator at 20% amplitude. Between each of these cycles, we allowed the sample to cool for two minutes. Following sonication, the exosomes were allowed to reintegrate their biological membranes via a 1-hour incubation at room temperature. After completion of the incubation, to separate the EGCG-loaded exosomes from naïve exosomes, ultracentrifugation was performed at $130,000 \times g$ (RCF) for 90 minutes, followed by pellet collection and redistribution of Exo-EGCG in PBS. The samples were subsequently processed for particle size measurement and then stored at -80 °C until use. To determine the encapsulation or entrapment efficiency, the EGCG-loaded exosomes were treated with methanol for 10 minutes and then centrifuged for 10 minutes at $10,000 \times g$ (RCF) to precipitate the lysed exosome particles. The supernatant containing the EGCG was subsequently analysed by UV analysis for EGCG concentration determination via following formula.

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Amount of drug entrapped in exosomes}}{\text{Total Amount of drug added}} \times 100$$

$$\text{Drug Loading (\%)} = \frac{\text{Amount of drug entrapped in exosomes}}{\text{Total Amount of drug added and exosomes weight}} \times 100$$

Characterization:

Bovine milk exosomes and Exo-EGCG were evaluated for size distribution, PDI, and zeta potential utilizing a Malvern Zetasizer Lab (Worcestershire, United Kingdom). Their structural topography was examined using an HR-TEM. Size and charge profiling were performed by diluting 100 µL of the respective samples into 1 mL of PBS. For morphological preparation, a mixture of 5 µL sample and 1.5 mL PBS was clarified through a 0.25 µm syringe filter. For TEM analysis, 100 µL of the diluted preparation was applied to 400-mesh grids, allowed to dry overnight, and examined at an operating voltage of 100 kV.

Drug release:

Following established methodologies [29], a dialysis-based dissolution study was conducted at pH 7.4 (representative of normal physiological blood) and pH 5.5 (representative of tumor acidity). Dialysis membranes (MWCO 14 kDa) were pre-conditioned overnight in PBS. Next, Exo-EGCG formulations (containing the equivalent of 1 mg EGCG) were sealed inside the membranes and immersed in 50 mL of PBS containing 0.2% Tween 80 to establish sink conditions. Media sampling (1 mL per time point) was executed at designated intervals over 48 hours (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h), with immediate replacement using fresh vehicle. Quantitation of the released EGCG was performed using UV spectrophotometric analysis.

Stability

The short-term stability of exosomes and Exo-EGCG was monitored over a six-week period at -80°C. Aliquots were sampled weekly from deep storage to measure changes in drug content, particle size, polydispersity index (PDI), and zeta potential.

Cell line and culturing conditions

SKGT-4 and FLO-1 cell lines were maintained in F-12 and DMEM medium, respectively, and both cell lines were provided with 10% FBS and 1% penicillin-streptomycin (antibiotic antimycotic solution) and cultured in a humidified incubator at 37°C with 5% CO₂.

Cytotoxicity

The in vitro cytotoxicity of Exo, EGCG, and Exo-EGCG against SKGT-4 and FLO-1 cell lines was determined via the standard colorimetric MTT assay. Briefly, cells were inoculated into 96-well culture plates at a seeding density of 1x10⁴ cells per well and incubated overnight at 37 °C to facilitate adherence. Following this 24 h stabilization period, the existing culture medium was discarded and replenished with fresh media containing gradient concentrations of Exo, EGCG, or Exo-EGCG. The treated cells were then incubated for designated intervals of 24, 48, and 72 h.

Upon completion of the respective exposure times, the treatment media were aspirated, and the cell monolayers were rinsed with PBS. Each well was then treated with 100 µL of MTT solution (0.5 mg/mL) and incubated for 4 h. Post-incubation, the supernatant was carefully removed, and the resulting intracellular formazan precipitates were solubilized by the addition of 100 µL of culture-grade DMSO, followed by a 30-minute incubation. The absorbance of the dissolved crystals was recorded at a wavelength of 570 nm using a microplate reader (EPOCH™, BioTek, Winooski, VT, USA). Cell viability percentages and corresponding half-maximal inhibitory concentrations IC₅₀ were modeled and computed using GraphPad Prism software (Boston, USA).

Qualitative cell uptake assay

The intracellular internalization of drug-loaded nanocarriers was qualitatively evaluated in SKGT-4 and FLO-1 esophageal cancer cells using coumarin-6 (C-6) as a fluorescent marker, adapting a previously reported protocol [30]. Fluorescently labeled exosomes (Exo-C-6) were synthesized using a methodology identical to Exo-EGCG, substituting EGCG with C-6. Cells were inoculated into 6-well plates at a density of 5×10^4 cells per well and cultured overnight to achieve adherence. Next, the cell monolayers were exposed to Exo-C-6 ($3 \mu\text{g/ml}$) for a duration of 4 h. Following incubation, the cells were rinsed thrice with ice-cold PBS to eliminate extracellularly bound or unencapsulated vesicles. The intracellularly accumulated C-6 fluorescence within the cancer cells was subsequently imaged and analyzed using a fluorescence microscope (Leica, Wetzlar, Germany).

Mitochondrial membrane potential assay

To evaluate the generation of mitochondrial reactive oxygen species under treatment conditions, mitochondrial superoxide levels were quantified using a commercial fluorometric assay kit (ab219943, Abcam, UK). Briefly, SKGT-4 and FLO-1 cells were seeded into 6-well plates at a density of 1×10^5 cells per well and allowed to adhere overnight under standard incubation conditions (37°C , 5% CO_2). The culture medium was subsequently replaced with fresh media containing free Exo, native EGCG, or Exo-EGCG at concentrations equivalent to the therapeutic dose of EGCG. Following a 24-hour treatment period, the cells were washed and incubated with the live-cell permeant MitoROS™ 580 dye solution for 1 hour at 37°C , strictly following the manufacturer's instructions. The specific oxidation of the dye by mitochondrial superoxide molecules yielded a distinct red fluorescent signal, which was immediately visualized and analyzed using a fluorescence microscope to evaluate intra-mitochondrial oxidative stress.

Reactive oxygen species assay

The accumulation of intracellular global reactive oxygen species (ROS) following formulation treatment was quantified using a commercial Cellular ROS Assay Kit (ab186029, Abcam, UK). Briefly, SKGT-4 and FLO-1 cell lines were seeded into 6-well plates at an initial density of 1×10^5 cells per well and cultured overnight under standard environmental conditions (37°C 5% CO_2) to ensure stable attachment. The old media was subsequently replaced with fresh culture media containing free Exo, native EGCG, or Exo-EGCG formulations, with drug concentrations normalized to the therapeutic equivalent dose of EGCG. Following a 24-hour treatment period, the cells were washed with PBS and incubated with the live-cell permeant ROS Deep Red dye solution for 45 minutes at 37°C in the dark, adhering closely to the manufacturer's assay protocol. Intracellular oxidation of the fluorogenic sensor by native ROS molecules generated a highly stable deep-red fluorescent signal. The relative intensity of this intracellular far-red fluorescence was immediately captured and analyzed using a fluorescence microscope to determine the extent of cellular oxidative stress.

Colony formation assay

In vitro cell survival and proliferation were monitored via a colony formation assay using SKGT-4 and FLO-1 cell lines. Individual cells (100 per dish) were distributed into 60 mm Petri dishes and cultured overnight at 37°C . After 24 h, the supernatant was evacuated and fresh media supplemented with Exo, EGCG, and Exo-EGCG (equivalent to $20 \mu\text{M}$ EGCG) was introduced. For the remaining duration of the 10-day experiment, the media was swapped for fresh regular medium every two days. On day 10, cell colonies were stained using a 0.5% crystal violet solution and manually enumerated to evaluate survival rates.

Wound healing assay

To determine the impact of Exo, EGCG, and Exo-EGCG on cellular migration, a scratch assay was carried out according to literature methods [31]. SKGT-4 and FLO-1 cells were distributed into Ibidi 2-well culture inserts (Ibidi, Lochhamer Schlag, Gräfelfing, Germany) at a density of 5×10^4 cells/100 μ L (200 μ L total volume) and incubated for 12 h. Following cell attachment, a cell-free gap was generated by gently lifting the inserts with sterile forceps. Monolayers were washed with cold PBS and then incubated with the different formulations (standardized to 10 μ M EGCG based on prior optimization). The progression of wound closure was evaluated using an inverted microscope at 0, 12, and 24 h, and the corresponding percentage of migration area was calculated following formula and using ImageJ software.

$$\text{Rate of wound closure} = \frac{\text{Distance of wound closure}}{\text{Time (Hours)}}$$

$$\text{Distance of wound closing} = \text{Initial wound distance} - \text{final wound distance}$$

Transwell migration assay

A transwell assay was executed to determine whether the prepared exosome formulations could inhibit the migratory capacity of SKGT-4 and FLO-1 cells. The cells were pretreated for 1 h with exosomes, EGCG, and Exo-EGCG (15 μ M EGCG equivalent based on preliminary work) before being harvested with trypsin. Next, cells were suspended in serum-starved media and introduced into the upper inserts (Sigma-Aldrich, MA, USA) at a density of 4×10^4 cells/100 μ L (total volume 0.5 mL). To drive migration, media containing 10% FBS was placed in the bottom well. Following 24 h of incubation, the migrating cells on the underside of the membrane were immobilized using 4% formaldehyde and visualized with 0.5% toluidine blue staining. Migration levels were determined by counting the stained cells under an inverted microscope.

Statistical analysis

Statistical computations were executed using GraphPad Prism software (Version 8.0; San Diego, CA, USA), while image processing was conducted via ImageJ software (Bethesda, Maryland, USA). To evaluate potential variations across the mean values of three independent experimental replicates, a one-way analysis of variance (ANOVA) was performed. Statistical significance was predefined and established at $p < 0.05$.

Result and discussion

Isolation and Biochemical Characterization of Exosomes

Exosomes were successfully isolated from bovine milk and subsequently characterized to confirm their molecular profile. As shown in Figure 1, Western blot analysis verified the presence of the classical exosomal membrane markers CD63, CD81, Tsg101, and Alix within the protein lysates. To establish the purity of the isolation, samples were screened for non-exosomal markers; the complete absence of p-selectin, CD40, and beta 1-integrin on the vesicle surfaces confirmed that the isolated fractions were highly purified and devoid of contaminating multivesicular bodies.

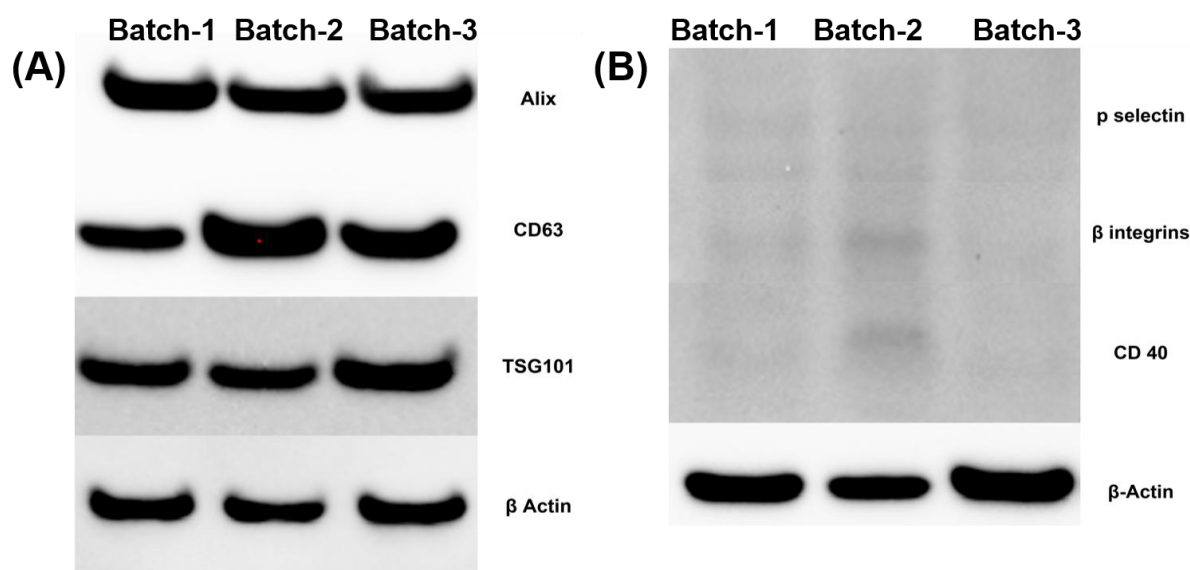


Figure 1: Validation of isolated vesicles. Exosomes were harvested across three independent batches using differential ultracentrifugation, with isolation purity confirmed via Western blot analysis of specific exosomal and microvesicle protein biomarkers.

Characterization of exosomes and drug -loaded exosomes

As summarized in Table II and illustrated in Figure 3(I)(a–c), the blank bovine milk exosomes displayed an average hydrodynamic diameter of 110.4 ± 2.5 nm, a polydispersity index (PDI) of 0.113 ± 0.003 , and a surface charge of -22.6 ± 2.5 mV. In comparison, the drug-loaded formulations (Exo-EGCG) exhibited a minor increase in size to 130.6 ± 3.6 nm, with a PDI of 0.108 ± 0.004 and a zeta potential of -24.5 ± 3.8 mV [Fig. 2 (A–D) and Table 1]. Although the increase in diameter was not statistically significant, these shifts are consistent with previous literature and point toward the successful incorporation of the therapeutic payload into or onto the exosomal vesicles [32]. Both systems demonstrated excellent colloidal stability; notably, Exo-EGCG possessed a slightly more pronounced negative surface charge than its native counterparts. This alteration in surface chemistry likely stems from the interaction of specific functional groups on the payload with the exosomal membrane via hydrogen bonding or electrostatic interactions.

Quantitative analysis revealed an encapsulation efficiency of $78.5 \pm 5.6\%$ and a drug loading capacity of $22.6 \pm 2.6\%$ (Table I). Morphological assessment via TEM confirmed that both unmodified and loaded exosomes maintained a well-defined spherical architecture. The physical diameters resolved by electron microscopy were roughly 80 nm, which is characteristically smaller than the dimensions obtained from dynamic light scattering (DLS). This variation is expected, as DLS measures the surrounding hydrodynamic hydration shell in a liquid state, whereas TEM captures the actual dehydrated core of the particles. Ultimately, payload loading did not compromise the structural integrity of the vesicles, confirming their viability as a stable drug delivery vehicle.

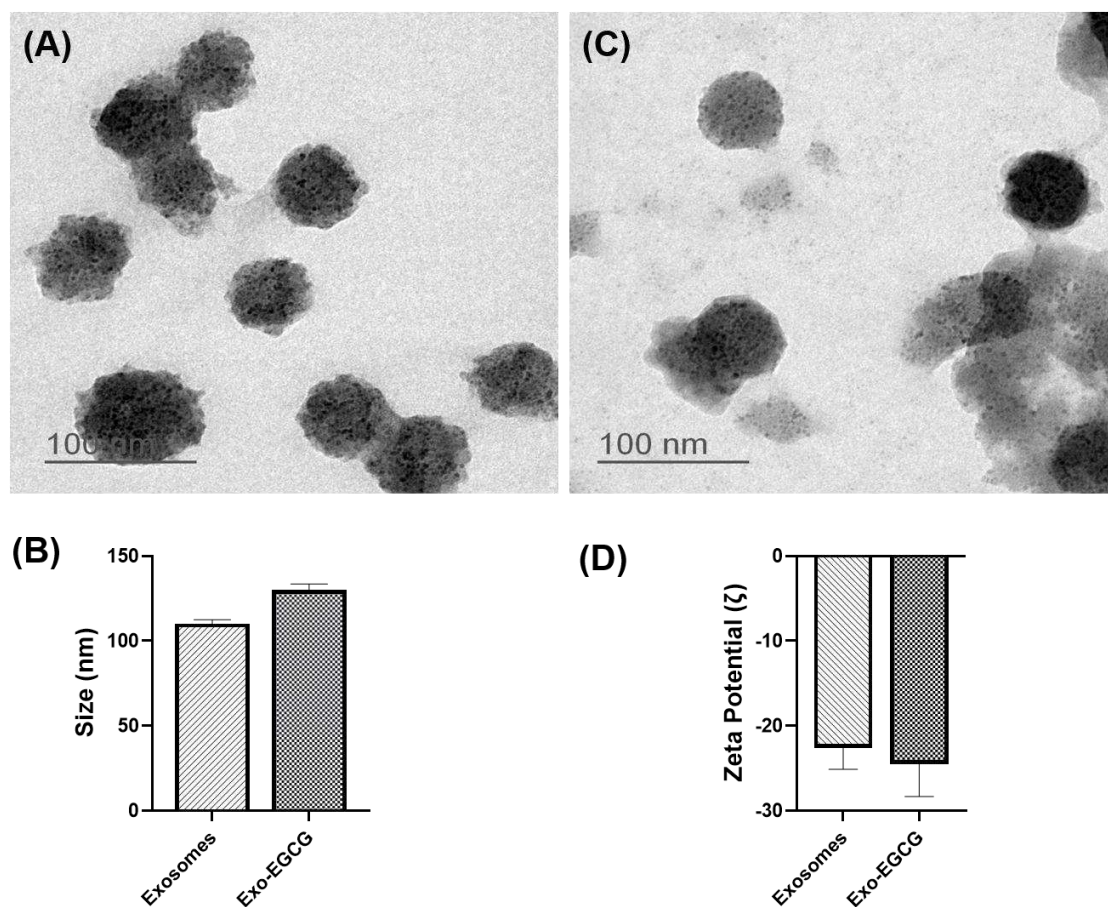


Figure 2: Morphological and structural characterization of native and drug-loaded vesicles. The shape and core dimensions of both unmodified exosomes (Exo) and epigallocatechin-3-gallate-loaded exosomes (Exo-EGCG) were visualized via transmission electron microscopy (TEM) (A), while their respective hydrodynamic size distributions were quantified using a dynamic light scattering particle size analyzer (B).

Table I: Physicochemical properties of native and drug-loaded vesicles. The table summarizes the hydrodynamic diameter, polydispersity index (PDI), surface charge (zeta potential), payload entrapment efficiency, and total drug loading capacity for both unmodified exosomes and epigallocatechin-3-gallate-encapsulated formulations.

Formulation	Size (nm)	PDI	Zeta potential	%EE	% DL
Exosomes	110.4±2.5	0.113±0.003	-22.6±2.5	-	-
Exo-EGCG	130.6±3.6	0.108±0.004	-24.5±3.8	78.5±5.6	22.6±2.6

Drug release

The *in vitro* cumulative release kinetics of EGCG and Exo-EGCG were investigated under dual environmental conditions to simulate systemic blood circulation (pH 7.4) and the acidic tumor interstitial fluid (pH 5.5). As illustrated in Figure 3 (A & B), Exo-EGCG displayed a characteristic biphasic release trajectory. Free EGCG exhibited rapid diffusion, achieving complete dissolution within 8 h across both pH media. Conversely, Exo-EGCG demonstrated an initial burst release during the first 2 h, with roughly 28% of the loaded cargo liberated irrespective of pH, followed by a prolonged, steady-state leakage extending up to 48 h. By the conclusion of the 48 h window, cumulative EGCG liberation reached approximately 80% at pH 7.4 and 95% at pH 5.5. The primary burst phase is likely governed by fractionally unencapsulated EGCG that adsorbed onto the vesicular surface through hydrogen bonds or hydrophobic interactions during the formulation phase. In contrast, the subsequent sustained phase is mediated by the slow partition of EGCG partitioned deep within the exosomal core. This dual-phase kinetic profile highlights the versatility of this platform for both immediate and extended therapeutic intervention against malignancy. Furthermore, the accelerated desorption observed at pH 5.5 can be attributed to the structural destabilization of the exosomal lipid bilayer under acidic stress, an effect modulated by surface-associated proteins and peptidoglycans native to the simulated tumoral microenvironment.

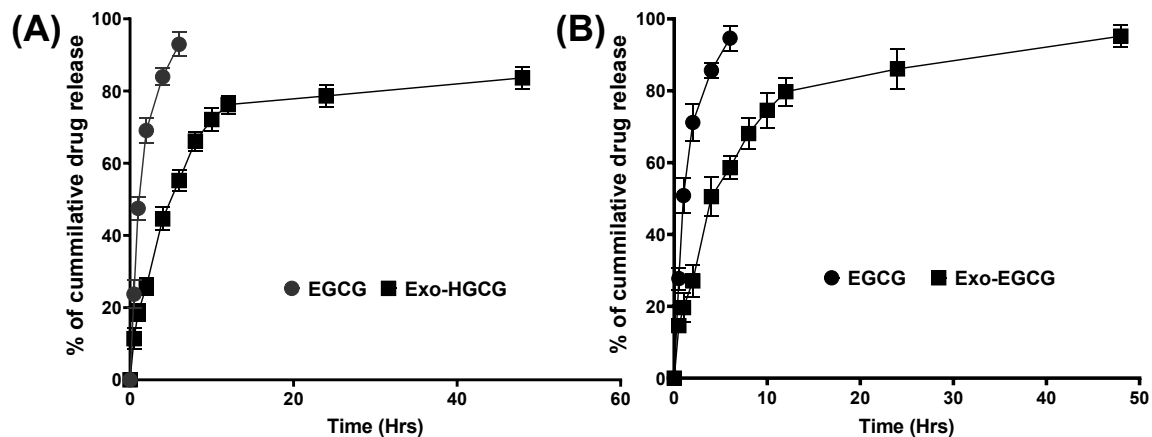


Figure 3: *In vitro* cumulative release kinetics of epigallocatechin-3-gallate from the exosomal carrier. The dissolution profiles were evaluated under dual experimental conditions to track cargo liberation at physiological blood parameters ((A) pH 7.4) versus simulated acidic tumor microenvironments ((B) pH 5.5).

Stability

The physical and chemical stability of unmodified exosomes and Exo-EGCG was evaluated over a five-week (one-month) storage period at an ultra-low temperature (-80°C) by monitoring changes in hydrodynamic size, PDI, and zeta potential. As illustrated in Figure 4, no significant alterations were detected in either the vesicle size or the remaining drug content for both groups. Parallel trends were observed regarding surface charge (zeta potential) and polydispersity indices. These minimal variations demonstrate that the exosomal formulations possess excellent structural integrity during short-term deep-freeze preservation. Nevertheless, comprehensive long-term stability trials monitoring both carrier degradation and precise cargo leakage remain essential to definitively validate the shelf-life of these systems.

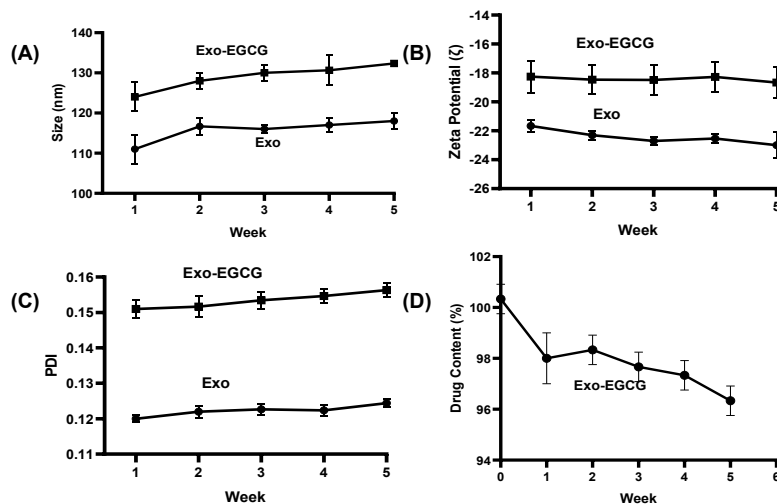


Figure 4: Short-term storage stability metrics for native and drug-loaded nanovesicles. The plots illustrate the impact of conservation time under ultra-low temperature preservation on fundamental colloidal and chemical parameters, including physical hydrodynamic diameter (A), net surface charge or zeta potential (B), polydispersity indices (C), and residual therapeutic cargo retention (D).

Cytotoxicity

To determine the anti-proliferative efficacy of the prepared formulations, the *in vitro* cytotoxicity of free EGCG and Exo-EGCG was monitored against SKGT-4 and FLO-1 cell lines using the metabolic MTT assay. This colorimetric method reflects cellular viability based on the enzymatic reduction of the MTT tetrazolium substrate into insoluble purple formazan crystals within metabolically active cells. Cell death or impaired proliferation is indicated by a reduction in crystal formation, enabling a direct correlation between optical density and cell survival. Dose- and time-dependent survival kinetics were tracked across 24, 48, and 72 h intervals, as illustrated in Figure 5 (I–II).

After 24 h of exposure in the SKGT-4 line, the calculated half-maximal inhibitory concentrations IC_{50} for free EGCG and Exo-EGCG were $72.2 \pm 2.1 \mu M$ and $41.5 \pm 3.2 \mu M$, respectively [Fig. 5(I)(A), Table III]. Prolonging the treatment window to 48 h triggered a distinct decline in the IC_{50} thresholds, yielding values of $48.4 \pm 2.8 \mu M$ for EGCG and $30.6 \pm 2.9 \mu M$ for Exo-EGCG [Fig. 5(I)(A–C), Table II]. This downward trend in IC_{50} values persisted through the 72 h endpoint. Comprehensive IC_{50} values across both cell models are compiled in Table III. Parallel cytotoxicity trends were resolved in the FLO-1 cell line under identical experimental parameters [Fig. 5(II)(A–D), Table II]. Notably, the SKGT-4 cells exhibited marginally higher resistance (increased IC_{50} profiles) relative to the FLO-1 line, an observation potentially tied to the pronounced cellular heterogeneity and highly aggressive biological phenotype characteristic of SKGT-4 cells. Crucially, the enhanced cytotoxic potency observed for EGCG upon exosomal encapsulation across both models is likely driven by carrier-mediated endocytosis, facilitating superior intracellular internalization of the polyphenol into the target cytoplasm or nuclear compartment.

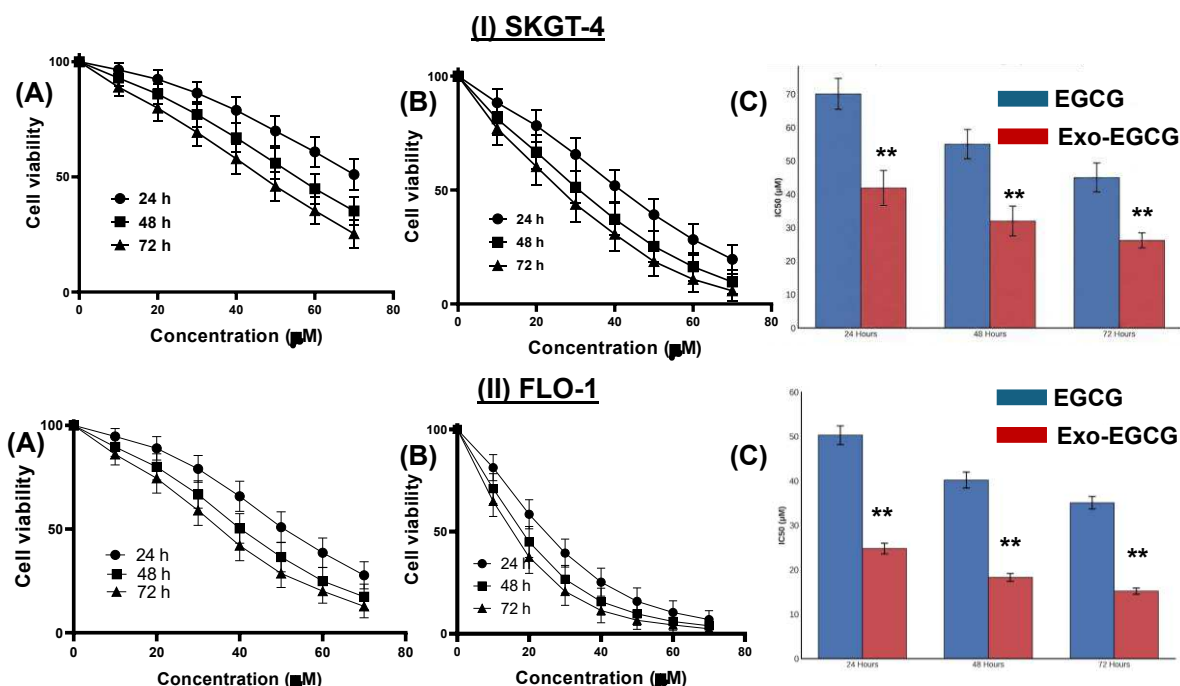


Figure 5: Evaluation of anti-proliferative activity against SKGT-4 and FLO-1 cell lines. The panel sets depict metabolic survival indices determined via MTT assay following exposure to variable drug formats over 24, 48, and 72 hours. **Panel I (SKGT-4):** Chronological IC₅₀ curves for native EGCG (a), exosome-encapsulated EGCG (b), and their superimposed comparison plots (c). **Panel II (FLO-1):** Parallel chronological IC₅₀ tracking for unencapsulated EGCG (a), Exo-EGCG (b), and the collective therapeutic comparison matrix (c). All data are expressed as mean $\pm$ SD (n = 3); statistical variations were evaluated via one-way ANOVA, where $*p < 0.05$ indicates statistical significance.

Table II: Half-maximal inhibitory concentration (IC₅₀) metrics of free and exosome-encapsulated epigallocatechin-3-gallate. The data summarizes the calculated cytotoxicity thresholds obtained in human SKGT-4 and FLO-1 cell lines across sequential incubation periods (24, 48, and 72 hours) to evaluate vehicle-mediated therapeutic enhancement.

Formulation	IC ₅₀ (μM)					
	24 Hours		48 Hours		72 Hours	
	SKGT-4	FLO-1	SKGT-4	FLO-1	SKGT-4	FLO-1
EGCG	71.2 $\pm$ 2.1	50.3 $\pm$ 2.1	48.4 $\pm$ 2.8	40.2 $\pm$ 1.8	41.5 $\pm$ 2.9	35.1 $\pm$ 1.4
Exo-EGCG	41.5 $\pm$ 3.2	24.8 $\pm$ 1.2	30.6 $\pm$ 2.9	18.3 $\pm$ 0.9	25.7 $\pm$ 3.1	15.2 $\pm$ 0.7

Cell uptake assay

To evaluate the capacity of the exosomal vehicles to internalize into esophageal cancer cells and transport their payload into the intracellular environment, a qualitative uptake study was performed in FLO-1 and SKGT-4 cell lines. Because the active therapeutic drug does not possess intrinsic fluorescence, the hydrophobic fluorophore coumarin-6 (C-6) was encapsulated as a surrogate tracker to generate fluorescently labelled vesicles (Exo-C-6). As illustrated in Figure 6 (A–D), cells treated with Exo-C-6 displayed a remarkably higher intensity of green fluorescence within the cytoplasm compared to those exposed to equivalent concentrations of free C-6. In contrast, control cells maintained in vehicle-only medium showed no green signal, confirming that the observed fluorescence was a direct consequence of carrier-mediated C-6 internalization. Interestingly, the intracellular accumulation of coumarin was visibly more pronounced in FLO-1 cells than in the SKGT-4 line [Fig. 6 (I–II)], which may suggest a potential P-glycoprotein or alternative efflux mechanism driving C-6 clearance in SKGT-4 cells, or reflect differences in their aggressive phenotype. Furthermore, counterstaining the nuclei with DAPI verified that the green signal was distributed intracellularly rather than merely adsorbed onto the outer plasma membrane. These internalization dynamics are highly consistent with previous literature reporting exosome-mediated drug delivery.

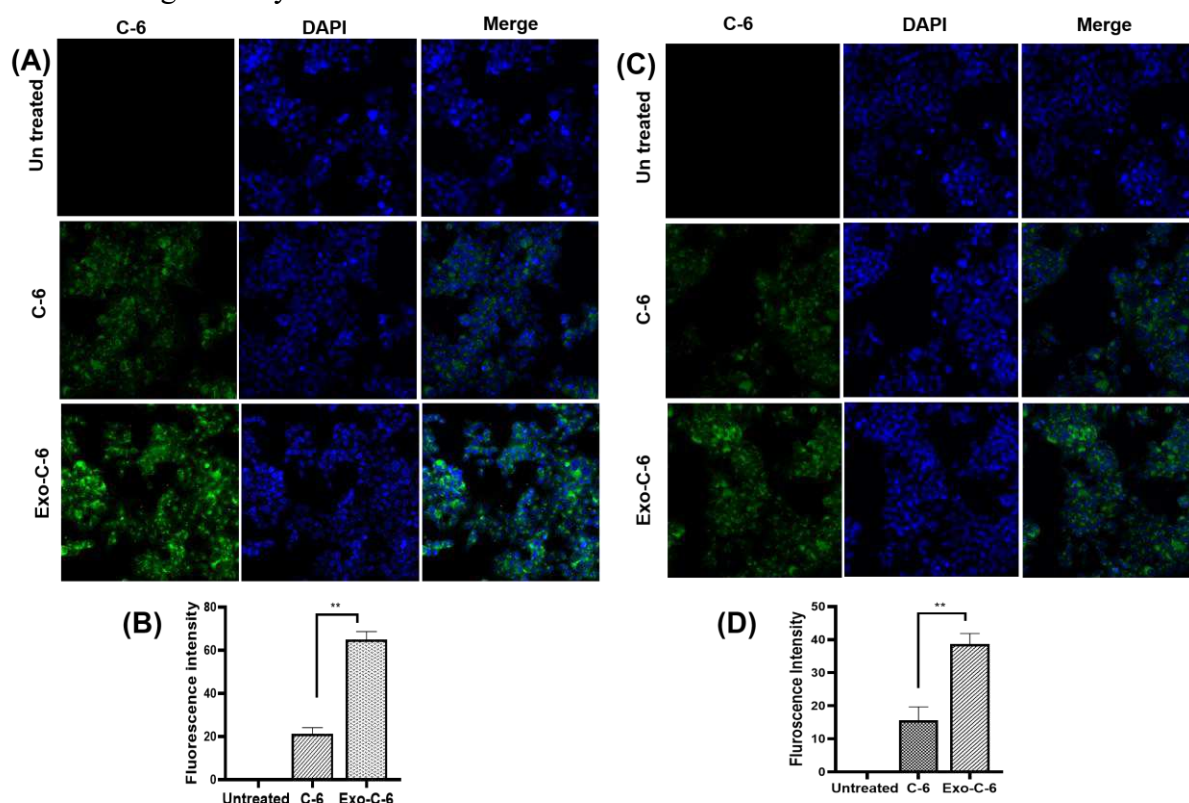


Figure 6: Microscopic evaluation of intracellular internalization kinetics in esophageal adenocarcinoma models. (A,C) Representative fluorescence microscopy images capturing the qualitative cellular uptake of free Coumarin-6 (C-6) versus exosome-encapsulated Coumarin-6 (Exo-C-6) within SKGT-4 (top panel) and FLO-1 (bottom panel) cell lines. (B, D) Quantitative analysis of relative intracellular fluorescence intensities processed via ImageJ software to compare delivery efficiencies. All data are expressed as mean $\pm$ SD (n = 3);

statistical variations were evaluated via one-way ANOVA, where $*p < 0.05$ indicates statistical significance.

Mitochondrial membrane potential assay

The accumulation of mitochondrial reactive oxygen species (ROS), particularly superoxide radicals, serves as a pivotal upstream indicator of mitochondrial dysfunction and the induction of the intrinsic apoptotic pathway. In this study, localized mitochondrial superoxide generation was monitored utilizing the fluorogenic MitoROS™ 580 probe, which undergoes specific oxidation within stressed mitochondria to emit a distinct red fluorescent signal proportional to oxidative stress levels [33]. Fluorescence microscopy analysis revealed that SKGT-4 cells treated with Exo-EGCG exhibited a significantly higher intensity of red fluorescence ($p < 0.05$) compared to those exposed to unencapsulated EGCG, demonstrating a more profound induction of mitochondrial oxidative damage (Figure 7 I (A–E)).

Parallel evaluation in the FLO-1 cell line yielded matching trends, with the Exo-EGCG formulation triggering a robust surge in red fluorescence relative to the free drug control groups (Figure 7 II (A–E)). Notably, the generation of mitochondrial superoxide was more pronounced in the FLO-1 line than in the SKGT-4 line. This cell line-specific variance can be attributed to the unique metabolic profiles and baseline antioxidant capacities inherent to each cell model; FLO-1 cells likely possess a lower threshold for polyphenol-induced mitochondrial disruption, making them more susceptible to severe oxidative stress. Taken together, these findings confirm that exosomal encapsulation drastically amplifies the capacity of EGCG to induce targeted mitochondrial superoxide overproduction, effectively destabilizing the malignant mitochondrial network [34].

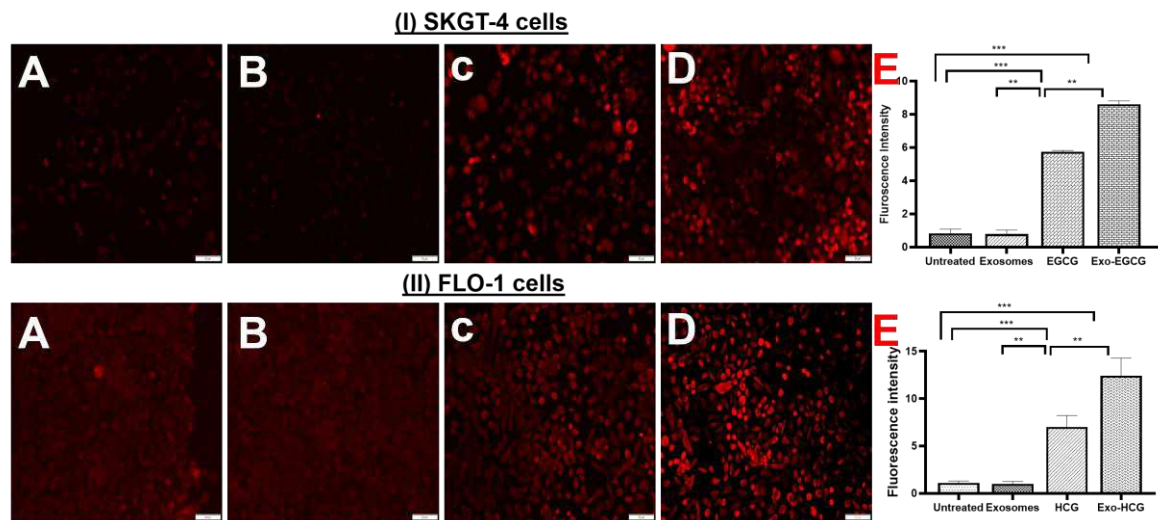


Figure 7: Assessment of mitochondrial membrane potential ($\Delta\psi_m$) variations in esophageal adenocarcinoma models following therapeutic exposure. (I) SKGT-4 cell cohort: Microscopic visualization of mitochondrial polarization profiles across experimental arms including (A) untreated controls, (B) blank exosome vehicles, (C) native unencapsulated epigallocatechin-3-gallate, and (D) cargo-loaded Exo-EGCG formulations. Relative fluorescence shifts indicating structural mitochondrial damage were quantified utilizing ImageJ processing software (E). (II) FLO-1 cell cohort: Parallel qualitative imaging and software-assisted fluorescence intensity measurements representing the respective treatments (A–E). Columns express mean values $\pm$ SD ($n = 3$); statistical margins were resolved via one-way ANOVA followed by Tukey's post-hoc test ($*p < 0.05$, $* * p < 0.01$, and $* * * p < 0.001$).

Reactive oxygen species assay

The accumulation of total intracellular reactive oxygen species (ROS) is a critical pathological event that compromises cellular homeostasis and acts as a major upstream trigger for oxidative stress-mediated apoptosis. In this study, global cytoplasmic ROS generation was tracked using a fluorogenic deep-red sensor that emits intense far-red fluorescence upon intracellular oxidation. Quantitative fluorescence microscopy revealed that SKGT-4 cells treated with Exo-EGCG exhibited a significantly higher intensity of deep-red fluorescence ($p < 0.05$) than those treated with free EGCG, proving that the nanocarrier substantially amplifies the intracellular oxidative burst (Figure 8 I (A–E)).

Parallel profiling in the FLO-1 cell line yielded matching trends, where the Exo-EGCG formulation successfully induced a massive surge in deep-red fluorescence relative to the free drug control groups (Figure 8 II (A–E)). When comparing the two esophageal cancer lines, the accumulation of cytoplasmic ROS was significantly more pronounced in FLO-1 cells than in the SKGT-4 line. This disparity is likely due to differences in endogenous antioxidant enzyme profiles; FLO-1 cells appear to have a lower capacity to neutralize exogenous pro-oxidant insults, making them exceptionally sensitive to exosome-mediated accumulation of intracellular free radicals. Together, these results confirm that exosomal encapsulation dramatically enhances the bio-delivery of EGCG, enabling it to overwhelm cellular antioxidant defenses and induce severe cellular oxidative damage.

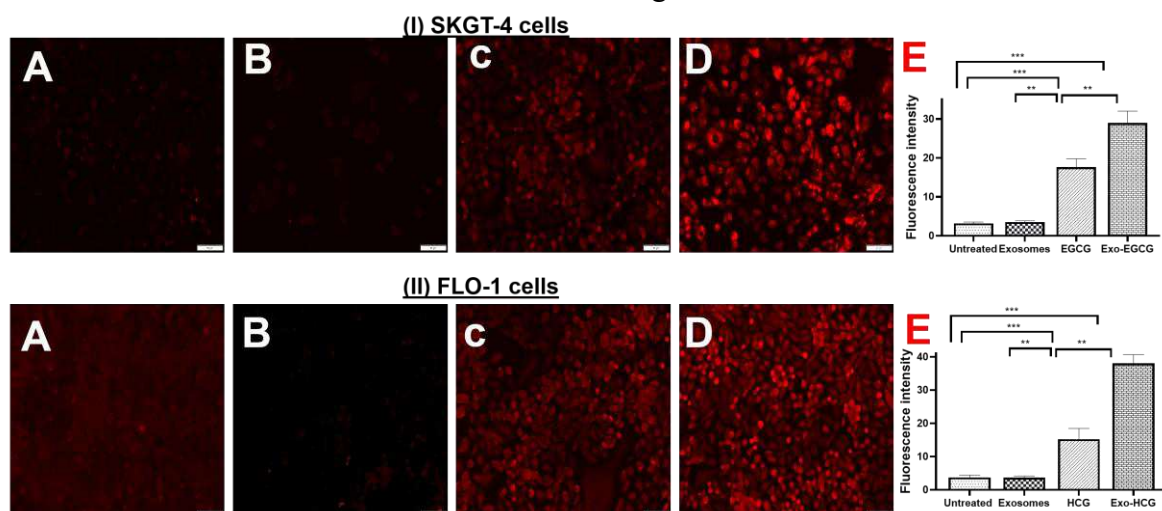


Figure 8: Evaluation of intracellular reactive oxygen species (ROS) accumulation in esophageal adenocarcinoma models following therapeutic exposure. (I) SKGT-4 cell cohort: Microscopic visualization of intracellular oxidative stress profiles using a fluorescent probe across experimental arms including (A) untreated controls, (B) blank exosome vehicles, (C) native unencapsulated epigallocatechin-3-gallate, (D) cargo-loaded Exo-EGCG formulations and (E) Relative fluorescence shifts indicating endogenous ROS generation were quantified utilizing ImageJ processing software (E). (II) FLO-1 cell cohort: Parallel qualitative imaging and software-assisted fluorescence intensity measurements representing the respective treatments (A–E). Columns express mean values $\pm$ SD ($n = 3$); statistical margins were resolved via one-way ANOVA followed by Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Colony formation assay

Clonogenic potential, which determines the capacity of a single cell to undergo unchecked proliferation, serves as a pivotal metric for evaluating long-term tumor control and recurrence risk. In this study, we assessed the colony-forming efficiency of SKGT-4 and FLO-1 esophageal cancer cells following exposure to empty exosomes, free EGCG, and Exo-EGCG. As illustrated in Figure 9 (I–II), SKGT-4 cells treated with free EGCG developed a significantly higher number of colonies ($p < 0.05$) than those treated with the exosomal formulation (Exo-EGCG). A matching inhibitory pattern was observed in the FLO-1 cell line, where Exo-EGCG treatment also markedly restricted colony survival. Overall, the SKGT-4 line generated a greater total number of colonies across the treatment groups. This variation likely arises from the highly aggressive phenotype of SKGT-4 cells, which exhibit a shorter doubling time relative to the FLO-1 line. Collectively, these data indicate that the encapsulation of EGCG within exosomal carriers significantly enhances its capacity to suppress single-cell reproductive survival, highlighting Exo-EGCG as a superior therapeutic strategy for preventing cancer recurrence compared to the unencapsulated polyphenol.

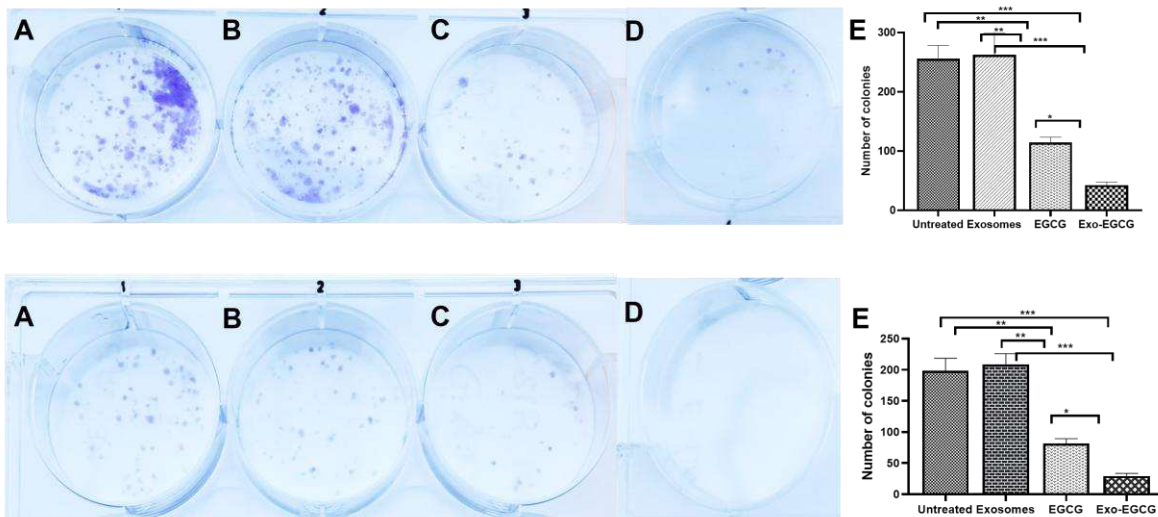


Figure 9: Assessment of long-term clonogenic survival capacity in esophageal adenocarcinoma models following therapeutic exposure. **(I) SKGT-4 cell cohort:** Representative macroscopic views of cell colonies establishing proliferative capacity under various experimental setups: (A) untreated control conditions, (B) blank exosome vehicles, (C) free unencapsulated epigallocatechin-3-gallate, and (D) cargo-loaded Exo-EGCG formulations. Relative colony survival densities were quantified utilizing ImageJ processing software (E). **(II) FLO-1 cell cohort:** Parallel clonogenic profiles and software-assisted density measurements representing the respective treatment arms (A-E). Columns express mean values $\pm$ SD ($n = 3$); statistical margins were resolved via one-way ANOVA followed by Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Migration assay

EAC, an aggressive malignancy with a rising global incidence, carries an exceptionally high risk of early systemic metastasis. The metastatic cascade in EAC is orchestrated by diverse cellular pressures, including localized hypoxia, nutrient deprivation, and the activation of specific pro-migratory signaling pathways. To evaluate the therapeutic potential of free EGCG and exosome-encapsulated EGCG (Exo-EGCG) in halting EAC cell motility, both transwell migration and scratch wound healing assays were performed utilizing the SKGT-4 and FLO-1 cell lines.

In contrast to the higher dosage (25 μ M) deployed in standard in vitro cell viability profiles, a sub-toxic concentration of EGCG (15 μ M) was selectively used for these migration challenges. This dosage optimization was informed by preliminary pilot investigations, where the 20 μ M threshold triggered direct cytotoxic cell death and structural morphological deformities that would confound migration kinetics. As demonstrated in Figure 10, cell translocation across both the untreated control and free EGCG-treated groups was significantly more pronounced ($p < 0.05$) than in the Exo-EGCG-treated cohorts.

Inter-cell line analysis revealed a distinct kinetic variation: the FLO-1 cells demonstrated accelerated migration rates leading to enhanced wound closure, whereas the treated SKGT-4 monolayers maintained a wider, less-healed wound gap, reflecting a more restrained migratory profile under these experimental parameters. Mechanistically, the marked suppression of transwell and lateral migration driven by Exo-EGCG can be attributed to the downregulation of the Focal Adhesion Kinase (FAK)/extracellular signal-regulated kinase (ERK) phosphorylation cascade, which is a principal pathway driving actin cytoskeleton remodeling and cellular protrusion in esophageal tumors [35, 36]. Furthermore, the enhanced delivery of EGCG via the exosomal framework likely suppresses the expression and enzymatic secretion of Matrix Metalloproteinases (specifically MMP-2 and MMP-9), thereby blocking the proteolytic degradation of the extracellular matrix required for invasive cell migration [37].

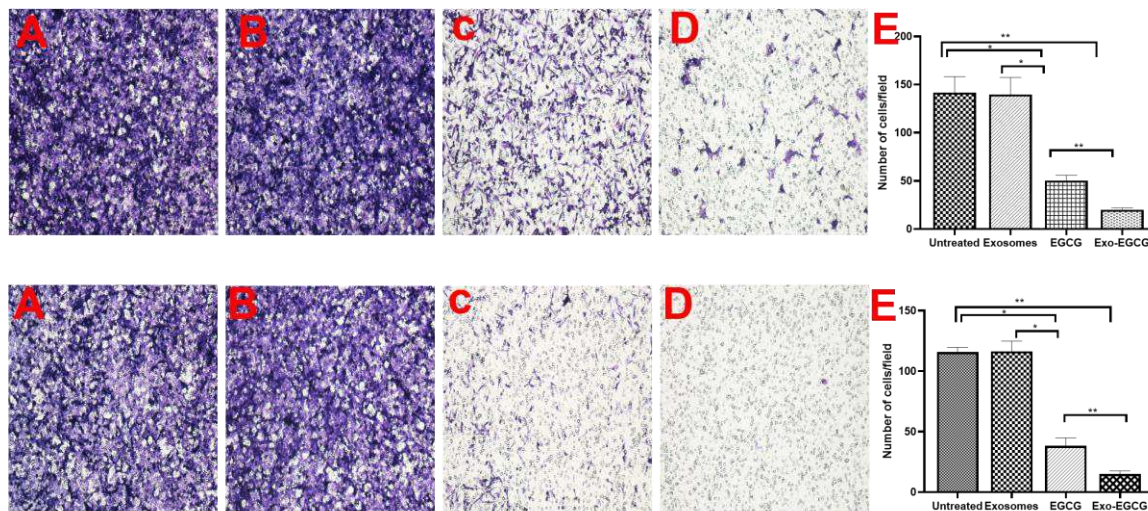


Figure 10 : Assessment of vertical cell migration capacity in esophageal adenocarcinoma models following therapeutic exposure. **(I) SKGT-4 cell cohort:** Representative microscopic views of migrated cells showing metastatic potential across experimental configurations: (A) untreated control conditions, (B) blank exosome vehicles, (C) free unencapsulated epigallocatechin-3-gallate, and (D) cargo-loaded Exo-EGCG formulations. The relative percentage of migrated cells was quantified utilizing ImageJ processing software (E). **(II) FLO-1 cell cohort:** Parallel migration profiles and software-assisted quantitative analysis representing the respective treatment arms (A–E). Columns express mean values $\pm$ SD ($n = 3$); statistical margins were resolved via one-way ANOVA followed by Tukey's post-hoc test ($*p < 0.05$, $* *p < 0.01$, and $* * *p < 0.001$).

Wound healing assay

To complement the transwell findings, a scratch wound healing assay was utilized in SKGT-4 and FLO-1 cell lines to further validate the anti-metastatic capabilities of free EGCG and Exo-

EGCG. As illustrated in Figure 10, the cellular migration required to close the wound gap was substantially decelerated in Exo-EGCG-treated groups relative to the free EGCG treatment. Consequently, cells exposed to the exosomal formulation recorded a drastically reduced percentage of wound closure ($p < 0.001$) alongside a diminished hourly closure rate compared to both control and free drug cohorts (Figure 11 I-II). Consistent with our earlier observations, cell line-specific variations were evident: FLO-1 cells exhibited a relatively higher degree of wound closure, whereas SKGT-4 cells demonstrated a much slower migratory response, leaving a larger unhealed area.

Synthesizing the data from both migration models confirms that Exo-EGCG potently impedes esophageal cancer invasion and expansion. Mechanistically, this suppression of motility is likely driven by the downregulation of critical pro-invasive markers, specifically matrix metalloproteinases (MMP-2, MMP-8, and MMP-9) and cyclooxygenase-2 (COX-2) [38]. It is widely recognized that the COX-2 and MMP families are indispensable for tumor angiogenesis, extracellular matrix degradation, and metastatic spread; our results align with this, indicating that targeted EGCG delivery effectively attenuates their expression [39]. Ultimately, these *in vitro* findings highlight that exosomal encapsulation drastically amplifies the anti-cancer potency of EGCG, though comprehensive *in vivo* models remain necessary to fully corroborate its therapeutic efficacy.

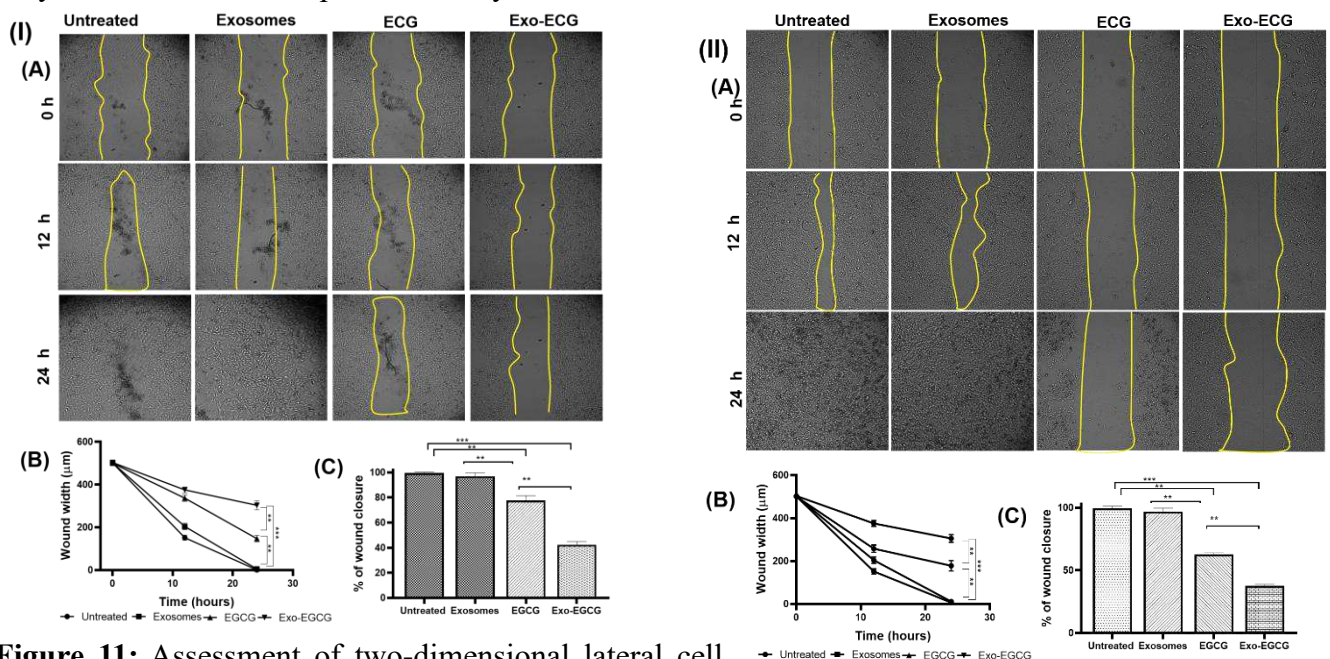


Figure 11: Assessment of two-dimensional lateral cell migration kinetics via scratch wound healing assays. **(I) SKGT-4 cell cohort:** (A) Representative phase-contrast micrographs capturing wound closure profiles at sequential chronological intervals (0, 12, and 24 hours) following treatment. (B) Mathematical tracking of residual wound width parameters across the temporal checkpoints. (C) Calculated percentage of absolute wound closure calculated at the 24-hour endpoint. **(II) FLO-1 cell cohort:** (A) Corresponding cell migration micrographs, (B) longitudinal scratch width kinetics, and (C) calculated percentage of wound closure metrics at 24 hours post-scratching for the respective treatment arms. Vertical columns represent mean values $\pm$ SD ($n = 3$); statistical significance across variations was mapped using a one-way ANOVA followed by Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Conclusion

This study demonstrates the successful development of a biocompatible, bovine milk-derived exosomal nanoplatfrom for the optimized delivery of the green tea polyphenol EGCG. The engineered Exo-EGCG formulations exhibit favourable physical properties, strong colloidal stability, and a distinct pH-dependent release profile that is accelerated within simulated tumor microenvironments. By utilizing exosome-mediated endocytosis, the formulation bypasses the inherent pharmacokinetic limitations of raw EGCG, leading to substantially enhanced cytotoxicity and a marked reduction in colony-forming efficiency across both SKGT-4 and FLO-1 esophageal adenocarcinoma models. Mechanistically, this therapeutic superiority is driven by a massive intracellular oxidative burst—evidenced by global cytoplasmic ROS accumulation and localized mitochondrial superoxide overproduction—as well as the potent suppression of key pro-metastatic and invasive pathways. While these *in vitro* findings highlight the exceptional promise of Exo-EGCG as a stable and scalable intervention to prevent tumor recurrence and systemic spread, further *in vivo* evaluation is necessary to fully validate its therapeutic efficacy and clinical translation potential.

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Conflict of interest

The authors confirm that no overlapping commercial interests or interpersonal relationships have exerted any influence over the research findings presented in this manuscript.

Data availability

Data will be made available on request from the authors.

References

- [1] H.G. Coleman, S.-H. Xie, J. Lagergren, The epidemiology of esophageal adenocarcinoma, *Gastroenterology* 154(2) (2018) 390-405.
- [2] P.C. Enzinger, R.J. Mayer, Esophageal cancer, *New England Journal of Medicine* 349(23) (2003) 2241-2252.
- [3] M. Pera, C. Manterola, O. Vidal, L. Grande, Epidemiology of esophageal adenocarcinoma, *Journal of surgical oncology* 92(3) (2005) 151-159.
- [4] R.L. Siegel, T.B. Kratzer, N.S. Wagle, H. Sung, A. Jemal, Cancer statistics, 2026, *Ca* 76(1) (2026) e70043.
- [5] D.J. Uhlenhopp, E.O. Then, T. Sunkara, V. Gaduputi, Epidemiology of esophageal cancer: update in global trends, etiology and risk factors, *Clinical journal of gastroenterology* 13(6) (2020) 1010-1021.
- [6] M. Sheikh, G. Roshandel, V. McCormack, R. Malekzadeh, Current status and future prospects for esophageal cancer, *Cancers* 15(3) (2023) 765.
- [7] C. Cabrera, R. Artacho, R. Giménez, Beneficial effects of green tea—a review, *Journal of the American College of Nutrition* 25(2) (2006) 79-99.
- [8] V. Sinija, H.N. Mishra, Green tea: Health benefits, *Journal of Nutritional & Environmental Medicine* 17(4) (2008) 232-242.
- [9] J. Schulze, L. Melzer, L. Smith, R. Teschke, Green tea and its extracts in cancer prevention and treatment, *Beverages* 3(1) (2017) 17.
- [10] S.-Y. Pan, Q. Nie, H.-C. Tai, X.-L. Song, Y.-F. Tong, L.-J.-F. Zhang, X.-W. Wu, Z.-H. Lin, Y.-Y. Zhang, D.-Y. Ye, Tea and tea drinking: China's outstanding contributions to the mankind, *Chinese medicine* 17(1) (2022) 27.

- [11] C. Schneider, T. Segre, Green tea: potential health benefits, *American family physician* 79(7) (2009) 591-594.
- [12] W.C. Reygaert, An update on the health benefits of green tea, *Beverages* 3(1) (2017) 6.
- [13] A. Basu, E.A. Lucas, Mechanisms and effects of green tea on cardiovascular health, *Nutrition reviews* 65(8) (2007) 361-375.
- [14] J. Pang, Z. Zhang, T.-z. Zheng, B.A. Bassig, C. Mao, X. Liu, Y. Zhu, K. Shi, J. Ge, Y.-j. Yang, Green tea consumption and risk of cardiovascular and ischemic related diseases: A meta-analysis, *International journal of cardiology* 202 (2016) 967-974.
- [15] J. Jankun, S.H. Selman, R. Swiercz, E. Skrzypczak-Jankun, Why drinking green tea could prevent cancer, *Nature* 387(6633) (1997) 561-561.
- [16] N.T. Zaveri, Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications, *Life sciences* 78(18) (2006) 2073-2080.
- [17] R.L. Pastore, P. Fratellone, Potential health benefits of green tea (*Camellia sinensis*): a narrative review, *Explore* 2(6) (2006) 531-539.
- [18] M. Qutub, U.M. Hussain, A. Tatode, T. Premchandani, R. Khan, M. Umekar, J. Taksande, P. Singanwad, Nano-engineered epigallocatechin gallate (EGCG) delivery systems: overcoming bioavailability barriers to unlock clinical potential in cancer therapy, *AAPS PharmSciTech* 26(5) (2025) 137.
- [19] A. Granja, M. Pinheiro, S. Reis, Epigallocatechin gallate nanodelivery systems for cancer therapy, *Nutrients* 8(5) (2016) 307.
- [20] M. Karimi-Shahri, A. Alalikhani, P. Hashemian, A. Hashemzadeh, H. Javid, The applications of epigallocatechin gallate (EGCG)-nanogold conjugate in cancer therapy, *Nanotechnology* 34(21) (2023) 212001.
- [21] K. Li, C. Teng, Q. Min, Advanced nanovehicles-enabled delivery systems of epigallocatechin gallate for cancer therapy, *Frontiers in chemistry* 8 (2020) 573297.
- [22] X. Peng, D.J. McClements, X. Liu, F. Liu, EGCG-based nanoparticles: synthesis, properties, and applications, *Critical reviews in food science and nutrition* 65(12) (2025) 2177-2198.
- [23] R. Munagala, F. Aqil, J. Jeyabalan, R.C. Gupta, Bovine milk-derived exosomes for drug delivery, *Cancer letters* 371(1) (2016) 48-61.
- [24] T.A. Reinhardt, J.D. Lippolis, B.J. Nonnecke, R.E. Sacco, Bovine milk exosome proteome, *Journal of proteomics* 75(5) (2012) 1486-1492.
- [25] A.M. Timofeeva, A.P. Paramonik, S.S. Sedykh, G.A. Nevinsky, Milk exosomes: next-generation agents for delivery of anticancer drugs and therapeutic nucleic acids, *International Journal of Molecular Sciences* 24(12) (2023) 10194.
- [26] D.N. Kumar, A. Chaudhuri, D. Dehari, A.M. Gamper, D. Kumar, A.K. Agrawal, Oral delivery of dihydroartemisinin for the treatment of melanoma via bovine milk exosomes, *Drug Delivery and Translational Research* 15(8) (2025) 2862-2877.
- [27] J.A. Welsh, D.C. Goberdhan, L. O'Driscoll, E.I. Buzas, C. Blenkiron, B. Bussolati, H. Cai, D. Di Vizio, T.A. Driedonks, U. Erdbrügger, Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches, *Journal of extracellular vesicles* 13(2) (2024) e12404.
- [28] D.N. Kumar, A. Chaudhuri, D. Dehari, A. Shekher, S.C. Gupta, S. Majumdar, S. Krishnamurthy, S. Singh, D. Kumar, A.K. Agrawal, Combination therapy comprising paclitaxel and 5-fluorouracil by using folic acid functionalized bovine milk exosomes improves the therapeutic efficacy against breast cancer, *Life* 12(8) (2022) 1143.
- [29] J. Pullan, K. Dailey, S. Bhallamudi, L. Feng, L. Alhalhooly, J. Froberg, J. Osborn, K. Sarkar, T. Molden, V. Sathish, Modified bovine milk exosomes for doxorubicin delivery to triple-negative breast cancer cells, *ACS applied bio materials* 5(5) (2022) 2163-2175.
- [30] Y.-K. Huang, M. Zhuang, M.A. Digman, FLIM intensity-based image segmentation reveals upregulated energy metabolism and chemotherapy sensitivity in MCF-7 cells, *Journal of Cell Science* 138(23) (2025) jcs263702.

- [31] J.T. Freitas, I. Jozic, B. Bedogni, Wound healing assay for melanoma cell migration, *Melanoma: Methods and Protocols*, Springer2021, pp. 65-71.
- [32] M.S. Kim, M.J. Haney, Y. Zhao, V. Mahajan, I. Deygen, N.L. Klyachko, E. Inskoe, A. Piroyan, M. Sokolsky, O. Okolie, Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells, *Nanomedicine: Nanotechnology, Biology and Medicine* 12(3) (2016) 655-664.
- [33] X. Yi, W. Guo, Q. Shi, Y. Yang, W. Zhang, X. Chen, P. Kang, J. Chen, T. Cui, J. Ma, SIRT3-dependent mitochondrial dynamics remodeling contributes to oxidative stress-induced melanocyte degeneration in vitiligo, *Theranostics* 9(6) (2019) 1614.
- [34] L. Tao, S.C. Forester, J.D. Lambert, The role of the mitochondrial oxidative stress in the cytotoxic effects of the green tea catechin, (–)-epigallocatechin-3-gallate, in oral cells, *Molecular nutrition & food research* 58(4) (2014) 665-676.
- [35] R. Wei, N.E. Cortez Penso, R.M. Hackman, Y. Wang, G.G. Mackenzie, Epigallocatechin-3-gallate (EGCG) suppresses pancreatic cancer cell growth, invasion, and migration partly through the inhibition of Akt pathway and epithelial–mesenchymal transition: Enhanced efficacy when combined with gemcitabine, *Nutrients* 11(8) (2019) 1856.
- [36] T. Li, N. Zhao, J. Lu, Q. Zhu, X. Liu, F. Hao, X. Jiao, Epigallocatechin gallate (EGCG) suppresses epithelial-Mesenchymal transition (EMT) and invasion in anaplastic thyroid carcinoma cells through blocking of TGF- β 1/Smad signaling pathways, *Bioengineered* 10(1) (2019) 282-291.
- [37] L.-C. Liu, T.C.-Y. Tsao, S.-R. Hsu, H.-C. Wang, T.-C. Tsai, J.-Y. Kao, T.-D. Way, EGCG inhibits transforming growth factor- β -mediated epithelial-to-mesenchymal transition via the inhibition of Smad2 and Erk1/2 signaling pathways in nonsmall cell lung cancer cells, *Journal of agricultural and food chemistry* 60(39) (2012) 9863-9873.
- [38] H. Tanabe, T. Suzuki, T. Ohishi, M. Isemura, Y. Nakamura, K. Unno, Effects of epigallocatechin-3-gallate on matrix metalloproteinases in terms of its anticancer activity, *Molecules* 28(2) (2023) 525.
- [39] F. Wang, Z. Chang, Q. Fan, L. Wang, Epigallocatechin-3-gallate inhibits the proliferation and migration of human ovarian carcinoma cells by modulating p38 kinase and matrix metalloproteinase-2, *Molecular Medicine Reports* 9(3) (2014) 1085-1089.