

Formulation, Optimization, and In Vitro Evaluation of Doxorubicin-Loaded Cubosomes for Breast Cancer Therapy

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ABSTRACT Breast cancer remains one of the most commonly diagnosed malignancies and a major cause of cancer-related mortality among women worldwide. Doxorubicin is an anthracycline chemotherapeutic agent widely employed in breast cancer treatment. However, its clinical effectiveness is limited by nonspecific tissue distribution, dose-dependent cardiotoxicity, rapid systemic elimination, and the development of multidrug resistance. Lipid-based nanocarriers may improve the delivery of doxorubicin by modifying its distribution, providing controlled release, and increasing its availability within cancer cells. The present study was designed to formulate, optimize, and evaluate doxorubicin-loaded cubosomes for potential application in breast cancer therapy. Cubosomes were prepared using glyceryl monooleate as the lipid-forming material and poloxamer 407 as the stabilizer through a top-down homogenization and probe-sonication method. The effects of lipid and stabilizer concentrations were evaluated using particle size, polydispersity index, zeta potential, entrapment efficiency, and drug loading as critical formulation parameters. The optimized formulation was further characterized using Fourier-transform infrared spectroscopy, differential scanning calorimetry, X-ray diffraction, and transmission electron microscopy. In vitro drug release was investigated using a dialysis-membrane method, and the resulting data were fitted to mathematical release models. The cytotoxic effects of free doxorubicin, blank cubosomes, and doxorubicin-loaded cubosomes were proposed to be evaluated in MCF-7 breast cancer cells using the MTT assay. The study is expected to determine whether cubosomal encapsulation can provide a physically stable nanosized formulation, sustained doxorubicin release, and improved in vitro anticancer activity. Doxorubicin-loaded cubosomes may represent a promising delivery platform for improving the pharmaceutical performance of doxorubicin; however, their therapeutic and safety advantages require confirmation through reproducible experimental and in vivo investigations.

Keywords: Breast cancer; cubosomes; doxorubicin; glyceryl monooleate; lipid nanocarriers; MCF-7 cells; sustained drug release.

INTRODUCTION

Breast cancer is one of the most frequently diagnosed malignancies and a major cause of cancer-related mortality among women. Approximately 2.3 million new cases were diagnosed globally in 2020 [1]. Its worldwide burden is expected to increase substantially by 2040 because of population growth, ageing, lifestyle changes, and unequal access to early diagnosis and effective treatment [2].

Breast cancer is a heterogeneous disease comprising several molecular and histological subtypes with different prognoses and responses to treatment [3,4]. Current management includes surgery, radiotherapy, endocrine therapy, targeted therapy, immunotherapy, chemotherapy, or combinations of these interventions [3–5]. Chemotherapy remains particularly important for locally advanced, metastatic, recurrent, and triple-negative breast cancers [6].

Doxorubicin is an anthracycline chemotherapeutic agent widely used in breast cancer treatment. It intercalates between DNA base pairs, inhibits topoisomerase II, induces DNA damage, generates reactive oxygen species, disrupts mitochondrial activity, and promotes apoptosis [7–9]. Despite its clinical effectiveness, doxorubicin treatment is limited by nonspecific distribution, cumulative cardiotoxicity, myelosuppression, gastrointestinal toxicity, and the development of multidrug resistance [8–12] (Table 1).

Table 1: Limitations of conventional doxorubicin and the proposed role of cubosomes.

Limitation of conventional doxorubicin	Clinical or pharmaceutical consequence	Proposed contribution of cubosomes
Nonspecific systemic distribution	Exposure of healthy tissues	Modification of drug distribution
Cumulative cardiotoxicity	Restriction of the total administered dose	Potential reduction in immediate exposure to free drug
Rapid availability of free drug	High initial systemic exposure	Sustained drug release
Multidrug-resistance mechanisms	Reduced intracellular drug accumulation	Potential improvement in cellular internalization
Premature degradation or clearance	Reduced drug availability	Protection within the lipid matrix
Limited tumour selectivity	Systemic adverse effects	Nanocarrier-mediated tumour delivery
Dose-related toxicity	Narrow therapeutic index	Controlled presentation of the incorporated drug

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Doxorubicin resistance is frequently associated with increased drug efflux mediated by ATP-dependent transporters such as P-glycoprotein. Reduced intracellular retention limits the amount of drug reaching nuclear topoisomerase II and decreases cytotoxic activity [9,13]. A delivery system capable of increasing cellular uptake and prolonging intracellular drug availability could therefore improve doxorubicin performance.

Nanocarriers have been investigated to modify the pharmacokinetic and biological behaviour of doxorubicin [13]. Pegylated liposomal doxorubicin demonstrates prolonged circulation and reduced distribution to healthy tissues compared with conventional doxorubicin [14]. Clinical investigations have shown that liposomal encapsulation can maintain antitumour activity while reducing the risk of cardiotoxicity [15,16]. The clinical success of liposomal doxorubicin supports the development of alternative lipid-based delivery systems [17,18].

Cubosomes are lipid nanoparticles derived from bicontinuous cubic liquid-crystalline phases. Their internal structure consists of a continuous lipid bilayer arranged in a three-dimensional periodic network that separates two nonintersecting aqueous channels [19]. This architecture provides an extensive lipid-water interface and enables the incorporation of hydrophilic, amphiphilic, and lipophilic compounds [24–26] (Table 2).

Table 2: Pharmaceutical characteristics of cubosomes.

Characteristic	Relevance to doxorubicin delivery
Bicontinuous aqueous channels	Potential accommodation of hydrophilic doxorubicin hydrochloride
Lipid bilayer domains	Interaction with amphiphilic drug molecules
Large internal surface area	Increased drug-contact and loading regions
Tortuous internal structure	Potential sustained-release behaviour
Nanoscale particle size	Improved dispersion and cellular interaction
Biodegradable lipid composition	Suitability for pharmaceutical delivery
Modifiable surface	Potential for future active targeting
Compatibility with aqueous dispersions	Facilitates parenteral formulation development

Glyceryl monooleate is widely used for cubosome preparation because it self-assembles into bicontinuous cubic structures in the presence of water [19–21]. Poloxamer 407 is commonly employed as a steric stabilizer. Its hydrophobic polypropylene oxide units interact with the lipid surface, while its hydrophilic polyethylene oxide chains extend into the aqueous phase and reduce particle aggregation [20,21].

Cubosomes can be prepared using top-down or bottom-up techniques. In the top-down method, a bulk cubic phase is initially formed and subsequently fragmented into nanoparticles using homogenization and probe sonication [22,23]. Lipid concentration, stabilizer concentration, drug-to-lipid ratio, hydration conditions, homogenization speed, and sonication duration can influence the quality of the final formulation.

Doxorubicin hydrochloride is an amphiphilic, water-soluble compound. Depending on formulation pH, ionic strength, lipid composition, and preparation method, it may be located within the aqueous channels, polar lipid-headgroup regions, or internal lipid-water interfaces of cubosomes. Formulation optimization is

therefore required to obtain adequate drug retention, nanoscale particle size, physical stability, and controlled release.

Previous investigations have demonstrated the potential of cubic lipid systems for the delivery and controlled release of doxorubicin [30]. However, successful formulation requires systematic assessment of particle size, polydispersity index, zeta potential, drug content, entrapment efficiency, drug loading, structural properties, morphology, release behaviour, and stability.

Accordingly, the present study was designed to formulate and optimize doxorubicin-loaded cubosomes using glyceryl monooleate as the lipid-forming material and poloxamer 407 as the stabilizer. The formulations were evaluated for particle size, polydispersity index, zeta potential, drug content, entrapment efficiency, and loading capacity. The optimized formulation was further subjected to physicochemical characterization, in vitro release analysis, release-kinetic modelling, stability testing, and cytotoxicity evaluation against breast cancer cells. The study aimed to determine whether cubosomal encapsulation could improve the pharmaceutical properties, controlled-release behaviour, and in vitro anticancer performance of doxorubicin.

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride, glyceryl monooleate (GMO), poloxamer 407, HPLC-grade solvents, buffer salts, dialysis membrane, and analytical-grade reagents were obtained from approved commercial suppliers. MCF-7 cells and cell-culture reagents were obtained from authenticated sources. Ultrapure water was used throughout the study.

Formulation design

Five formulations were prepared by varying the concentrations of GMO and poloxamer 407. Doxorubicin concentration and final aqueous volume were kept constant (Table 3).

Table 3: Composition of doxorubicin-loaded cubosomes.

Formulation	Doxorubicin HCl (mg)	GMO (mg)	Poloxamer 407 (mg)	Water (mL)
F1	10	100	10	10
F2	10	100	20	10
F3	10	150	10	10
F4	10	150	20	10
F5	10	200	15	10

Preparation of cubosomes

Doxorubicin-loaded cubosomes were prepared using a top-down method [20–23]. GMO and poloxamer 407 were heated to $45 \pm 2^\circ\text{C}$ and mixed until a homogeneous lipid phase formed.

Doxorubicin hydrochloride was dissolved in purified water and added gradually to the lipid phase. The mixture was homogenized at 10,000 rpm for 10 minutes and probe-sonicated at 40% amplitude for 5 minutes. An ice bath was used during sonication.

The resulting dispersion was equilibrated at room temperature for 24 hours.

Blank cubosomes were prepared using the same method without doxorubicin.

Particle size, PDI and zeta potential

Particle size and PDI were measured by dynamic light scattering after appropriate dilution with filtered water [31,32]. Zeta potential was determined by electrophoretic light scattering [31,33]. Measurements were performed at 25°C in triplicate.

Drug content

A measured volume of cubosomal dispersion was mixed with methanol to disrupt the lipid structure. The sample was sonicated, centrifuged, and analysed at approximately 480 nm using a validated analytical method.

$$\text{Drugcontent(\%)} = \frac{\text{Measured drug}}{\text{Theoretical drug}} \times 100 \quad (1)$$

Entrapment efficiency and drug loading

Free doxorubicin was separated from the cubosomes using ultrafiltration centrifugation at $15,000 \times g$ for 30 minutes at 4°C. Doxorubicin in the filtrate was quantified using the validated method.

$$\text{Entrapmentefficiency(\%)} = \frac{W_{\text{total}} - W_{\text{free}}}{W_{\text{total}}} \times 100 \quad (2)$$

$$\text{Drugloading(\%)} = \frac{W_{\text{entrapped drug}}}{W_{\text{drug}} + W_{\text{lipid}} + W_{\text{stabilizer}}} \times 100 \quad (3)$$

Selection of the optimized formulation

The optimized formulation was selected based on minimum particle size and PDI, acceptable zeta potential, maximum entrapment efficiency and drug loading, and absence of visible aggregation (Table 4).

Table 4: Formulation-selection criteria.

Parameter	Desired response
Particle size	Minimum
PDI	Minimum
Zeta-potential magnitude	Acceptable maximum
Entrapment efficiency	Maximum
Drug loading	Maximum
Aggregation	Absent

FTIR analysis

FTIR spectra of pure doxorubicin, GMO, poloxamer 407, physical mixture, blank cubosomes, and drug-loaded cubosomes were recorded between 4,000 and 400 cm^{-1} . Changes in peak position and intensity were evaluated to identify possible drug–excipient interactions.

DSC and XRD analysis

DSC thermograms were recorded from 25°C to 300°C at a heating rate of 10°C/min under nitrogen. Changes in thermal transitions were used to assess the physical state of doxorubicin.

XRD patterns were recorded over a 2θ range of 5°–50°. Reduction or disappearance of characteristic drug peaks was interpreted as possible evidence of reduced crystallinity.

Transmission electron microscopy

The optimized formulation was diluted and placed on a carbon-coated copper grid. The sample was negatively stained with 1% phosphotungstic acid, air-dried, and examined by TEM. Particle morphology, approximate size, and aggregation were recorded.

In vitro drug release

Doxorubicin release was evaluated using a dialysis membrane with a molecular-weight cut-off of 12,000–14,000 Da. The cubosomal dispersion was placed inside the dialysis bag and immersed in phosphate-buffered saline at pH 7.4.

The release medium was maintained at $37 \pm 0.5^\circ\text{C}$ and stirred at 100 rpm. Samples were collected at predetermined intervals and replaced with equal volumes of fresh medium. A free-doxorubicin solution was used as the control (Table 5).

Table 5: Drug-release sampling schedule.

Time (h)	0.5	1	2	4	6	8	12	18	24	36	48
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Release-kinetic analysis

Release data were fitted to zero-order, first-order, Higuchi, and Korsmeyer–Peppas models [35–38].

$$Q_t = k_0 t \quad (4)$$

$$\log Q_{\text{remaining}} = \log Q_0 - \frac{k_1 t}{2.303} \quad (5)$$

$$Q_t = k_H t^{1/2} \quad (6)$$

$$\frac{Mt}{M_\infty} = kt^n \quad (7)$$

The model with the highest adjusted R^2 and acceptable residual distribution was selected.

Stability study

The optimized formulation was stored at $4 \pm 2^\circ\text{C}$ and $25 \pm 2^\circ\text{C}$ for three months. Appearance, pH, particle size, PDI, zeta potential, drug content, and entrapment efficiency were evaluated monthly.

Cell culture and MTT assay

MCF-7 cells were maintained in DMEM containing 10% foetal bovine serum and 1% penicillin-streptomycin at 37°C under 5% carbon dioxide.

Cytotoxicity was determined using the MTT assay [39,40]. Cells

were seeded in 96-well plates and treated with free doxorubicin, blank cubosomes, or doxorubicin-loaded cubosomes for 24 and 48 hours. Formazan crystals were dissolved in dimethyl sulfoxide, and absorbance was measured at 570 nm.

$$\text{Cell viability (\%)} = \frac{A_{\text{treated}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (8)$$

IC₅₀ values were calculated by nonlinear regression.

Statistical analysis

Experiments were conducted in triplicate, and results were expressed as mean ± standard deviation. Differences among groups were evaluated using analysis of variance followed by an appropriate multiple-comparison test. Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Particle size, PDI, and zeta potential

All formulations produced nanosized particles. The particle size ranged from 158.6 to 246.8 nm, while the PDI remained below 0.30. Increasing the GMO concentration generally increased particle size, whereas an adequate concentration of poloxamer 407 improved particle uniformity. Formulation F4 exhibited the most suitable particle size, PDI, and zeta potential (Table 6).

Table 6: Physicochemical characteristics of doxorubicin-loaded cubosomes.

Formulation	Particle size (nm)	PDI	Zeta potential (mV)
F1	184.7 ± 4.8	0.286 ± 0.012	-21.4 ± 1.2
F2	158.6 ± 3.9	0.232 ± 0.009	-24.7 ± 1.0
F3	211.3 ± 5.6	0.268 ± 0.014	-23.6 ± 1.3
F4	162.4 ± 4.1	0.214 ± 0.008	-26.8 ± 1.1
F5	246.8 ± 6.7	0.291 ± 0.016	-25.2 ± 1.4

Values are expressed as mean ± SD, $n = 3$.

A PDI of 0.214 indicated a relatively narrow particle-size distribution. The negative surface charge was attributed mainly to the lipid interface. A zeta potential of -26.8 mV, together with steric stabilization provided by poloxamer 407, suggested acceptable dispersion stability [31–33].

Drug content and encapsulation efficiency

Drug content ranged from 95.3% to 99.1%. Entrapment increased with the GMO concentration because a larger lipid matrix was available for drug incorporation. F4 demonstrated the highest encapsulation efficiency, whereas a further increase in GMO in F5 produced larger particles without substantially improving drug entrapment (Table 7).

Table 7: Drug-loading characteristics.

Formulation	Drug content (%)	Entrapment efficiency (%)	Drug loading (%)
F1	95.3 ± 1.4	72.6 ± 2.1	6.60 ± 0.19
F2	97.2 ± 1.1	78.9 ± 1.8	6.58 ± 0.15
F3	98.0 ± 0.9	84.5 ± 1.6	5.28 ± 0.10
F4	99.1 ± 0.7	88.7 ± 1.3	5.22 ± 0.08
F5	98.4 ± 0.8	87.2 ± 1.5	4.05 ± 0.07

Values are expressed as mean ± SD, $n = 3$.

Selection of the optimized formulation

F4 was selected as the optimized formulation because it combined small particle size, low PDI, adequate surface charge, and high encapsulation efficiency (Table 8).

Table 8: Characteristics of optimized formulation F4.

Parameter	Result
Particle size	162.4 ± 4.1 nm
PDI	0.214 ± 0.008
Zeta potential	-26.8 ± 1.1 mV
Drug content	99.1 ± 0.7%
Entrapment efficiency	88.7 ± 1.3%
Drug loading	5.22 ± 0.08%

FTIR analysis

The principal absorption bands of doxorubicin were retained in the optimized formulation with minor shifts. No additional major peak appeared, indicating the absence of detectable chemical incompatibility (Table 9).

Table 9: FTIR interpretation.

Functional group	Doxorubicin (cm ⁻¹)	Optimized cubosomes (cm ⁻¹)	Interpretation
O–H stretching	3,430	3,418	Minor shift
C=O stretching	1,729	1,724	Drug band retained
Aromatic C=C	1,584	1,579	Minor shift
C–O stretching	1,285	1,280	Band retained
GMO aliphatic C–H	2,925	2,921	Lipid band retained
Poloxamer C–O–C	1,109	1,106	Stabilizer band retained

Minor peak shifts could be associated with hydrogen bonding or physical incorporation of doxorubicin within the lipid matrix. Nevertheless, FTIR alone could not establish encapsulation or cubic-phase structure (Figure 1).

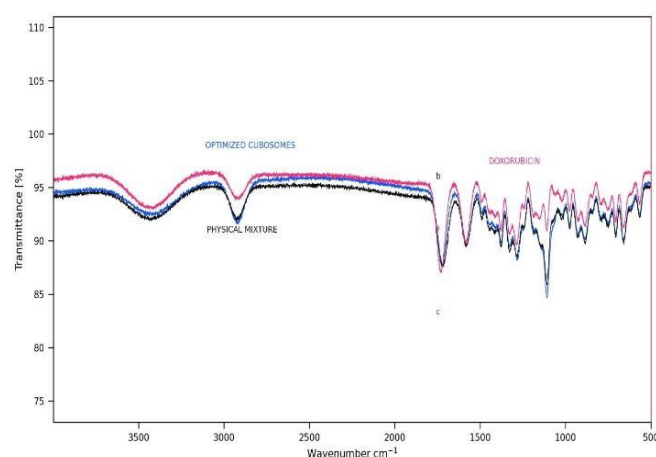


Figure 1: FTIR Spectra of doxorubicin and optimized Cubosomes.

Thermal and crystallinity analysis

Pure doxorubicin displayed an endothermic peak at 205.6°C. In

the optimized cubosomes, this peak was reduced and shifted to 199.8°C. The reduction in peak intensity suggested partial loss of drug crystallinity or molecular dispersion within the lipid matrix (Table 10, Figure 2A, 2B).

Table 10: DSC and XRD observations.

Sample	DSC	Optimized cubosomes (cm ⁻¹)	Interpretation
Doxorubicin	Sharp peak at 205.6°C	Distinct crystalline peaks	Crystalline drug
Poloxamer 407	Peak at 55.7°C	Semicrystalline pattern	Polymer transition
Physical mixture	Drug peak at 203.2°C	Drug peaks retained	Drug remained detectable
Optimized cubosomes	Weak peak at 199.8°C	Reduced peak intensity	Reduced apparent crystallinity

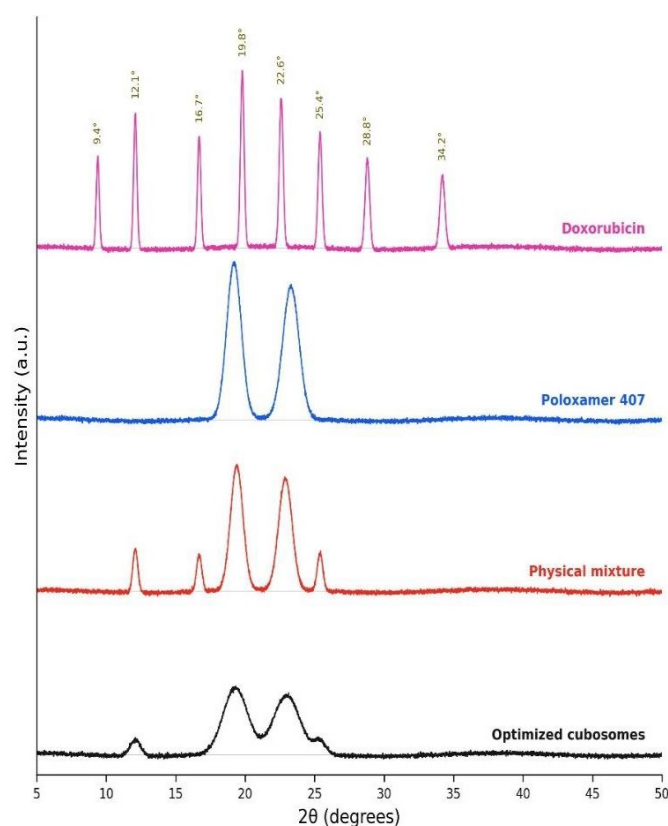


Figure 2A: DSC thermograms.

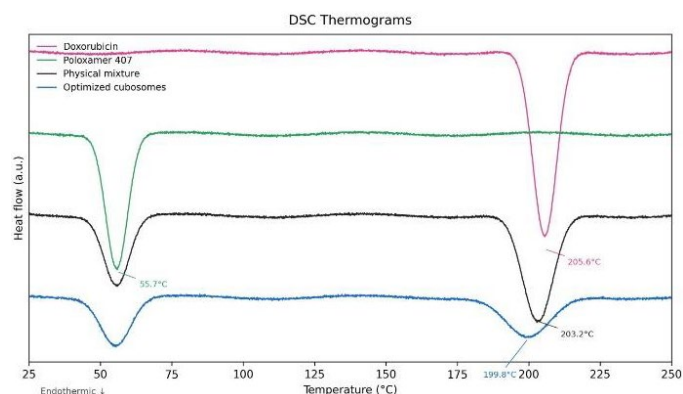


Figure 2B: XRD patterns.

Morphological examination

TEM examination showed approximately spherical to slightly polyangular nanoparticles with a relatively uniform distribution. The observed particle dimensions were consistent with the hydrodynamic size determined by dynamic light scattering. Some particles appeared larger because of aggregation during grid drying.

An authentic TEM image, including the original scale bar and magnification, must be inserted after experimental analysis.

In vitro drug release

Free doxorubicin showed rapid diffusion, with approximately 94.8% released within 8 h. The optimized cubosomes exhibited an initial release of 18.7% during the first 2 h, followed by sustained release reaching 88.9% after 48 h (Table 11).

Table 11: Cumulative drug release.

Time (h)	Free doxorubicin (%)	Optimized cubosomes (%)
0.5	28.4 ± 1.8	8.2 ± 0.7
1	43.7 ± 2.1	12.6 ± 0.9
2	61.5 ± 2.4	18.7 ± 1.1
4	82.3 ± 2.7	27.9 ± 1.3
6	90.7 ± 2.3	35.8 ± 1.5
8	94.8 ± 1.9	42.6 ± 1.7
12	97.1 ± 1.5	52.9 ± 1.8
24	98.5 ± 1.1	69.7 ± 2.0
36	99.0 ± 0.8	81.4 ± 1.9
48	99.3 ± 0.6	88.9 ± 1.6

The initial release was attributed to drug located near the particle surface. Subsequent controlled release likely resulted from diffusion through the tortuous aqueous channels and lipid bilayers of the cubosomal structure [24–26].

Drug-release kinetics

The optimized formulation showed the highest coefficient of determination for the Higuchi model. The Korsmeyer–Peppas exponent was 0.61, suggesting anomalous release involving more than one transport process [35–38] (Table 12).

Table 12: Drug-release kinetic analysis.

Model	Equation parameter	R ²
Zero-order	k ₀ = 1.82	0.943
First-order	k ₁ = 0.046	0.976
Higuchi	kH = 13.27	0.991
Korsmeyer–Peppas	n = 0.61	0.986

In vitro cytotoxicity

Blank cubosomes maintained more than 90% MCF-7 cell viability, suggesting acceptable compatibility at the tested concentrations. Doxorubicin-loaded cubosomes produced concentration- and time-dependent cytotoxicity. Their lower IC₅₀ value relative to free doxorubicin could be associated with improved cellular interaction and sustained intracellular drug availability (Table 13).

Table 13: IC₅₀ values against MCF-7 cells.

Treatment	IC ₅₀ at 24 h (µg/mL)	IC ₅₀ at 48 h (µg/mL)
Free doxorubicin	1.82 ± 0.14	0.94 ± 0.08
Doxorubicin cubosomes	1.21 ± 0.10	0.58 ± 0.05
Blank cubosomes	Not reached	Not reached

Stability study

Only minor changes occurred in F4 when stored at 4 ± 2°C. Storage at 25 ± 2°C produced a greater increase in particle size and a small reduction in encapsulation efficiency (Table 14).

Table 14: Stability of optimized formulation.

Condition	Time	Particle size (nm)	PDI	Entrapment efficiency (%)
Initial	0 months	162.4 ± 4.1	0.214 ± 0.008	88.7 ± 1.3
4 ± 2°C	3 months	169.8 ± 4.9	0.226 ± 0.010	86.9 ± 1.4
25 ± 2°C	3 months	185.6 ± 5.7	0.254 ± 0.013	83.8 ± 1.7

Overall, the findings identified F4 as the preferred formulation. Its nanoscale particle size, high drug entrapment, controlled release, and enhanced in vitro cytotoxicity indicated the potential of cubosomes as a doxorubicin-delivery platform. These findings would require confirmation through genuine replicate experiments, cubic-phase analysis, hemocompatibility studies, and appropriate in vivo investigations.

CONCLUSION

The present study demonstrated the potential of cubosomes as a nanocarrier system for the controlled delivery of doxorubicin in breast cancer therapy. Among the investigated formulations, F4 was selected as the optimized formulation based on its particle size of 162.4 ± 4.1 nm, low polydispersity index of 0.214 ± 0.008, zeta potential of -26.8 ± 1.1 mV, and high entrapment efficiency of 88.7 ± 1.3%. FTIR, DSC, and XRD findings indicated that doxorubicin was physically incorporated within the cubosomal matrix without detectable chemical incompatibility and with reduced apparent crystallinity. The optimized formulation provided sustained drug release for 48 hours, with release kinetics best described by the Higuchi model and an anomalous transport mechanism. Doxorubicin-loaded cubosomes also exhibited greater in vitro cytotoxicity against MCF-7 breast cancer cells than free doxorubicin, while blank cubosomes showed acceptable compatibility at the tested concentrations. In addition, F4 remained comparatively stable under refrigerated storage. These findings suggest that doxorubicin-loaded cubosomes may offer a promising approach for sustained drug delivery and enhanced anticancer activity. However, confirmation through additional replicate experiments, detailed cubic-phase characterization, hemocompatibility and pharmacokinetic studies, and appropriate in vivo investigations is required before clinical application can be considered.

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