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Dual drug-loaded cubosome nanoparticles for hepatocellular carcinoma: a design of experiment approach for optimization and in vitro evaluation

Poorvika Badiger[1], V. S. Mannur[1*] and Rahul Koli[1]

Abstract

Background Liver cancer, a formidable and complex disease, poses a significant global health threat, stemming from various causes, including chronic infections like hepatitis B and C, cirrhosis, and lifestyle factors. In liver cancer treatment, targeted delivery revolutionizes precision therapy, minimizing side effects by directing drugs specifically to cancer cells. This study aims to develop and statistically optimize cubosomal formulations containing piperine and quercetin with the goal of augmenting their activity against hepatocellular carcinoma.

Results Employing a central-composite design, we utilized Design-Expert® software to guide the experiment. The key formulation variables were the concentration of glyceryl monooleate (GMO) and Poloxamer-407, while the dependent responses were particle size (PS) and entrapment efficiency (EE%). The optimized cubosomal formulation was validated through the utilization of high-resolution transmission electron microscopy (HR-TEM), in vitro release studies, and an in vitro cell proliferation assay conducted on the HepG2 cell line. High-performance liquid chromatography was employed for the determination of piperine and quercetin in the optimized cubosomal nanoparticle. The optimized formulation had a composition of 2.5 (w/w%) GMO and 0.5 (w/w%) Poloxamer 407. The predicted values for PS and EE% were 102.34 and 75.11%, respectively. The cytotoxicity of the optimized cubosomal formulation exhibited enhanced efficacy on the HepG2 cancer cell line, even at lower concentrations, when compared to the standard. Notably, it demonstrated a superior cytotoxic effect on the liver cancer cell line.

Conclusion The findings of the study indicated that cubosomes exhibit promise as an effective carrier for delivering piperine and quercetin, addressing hepatocellular carcinoma effectively.

Keywords Piperine, Quercetin, Hepatocellular carcinoma, Quality by design, High-performance liquid chromatography

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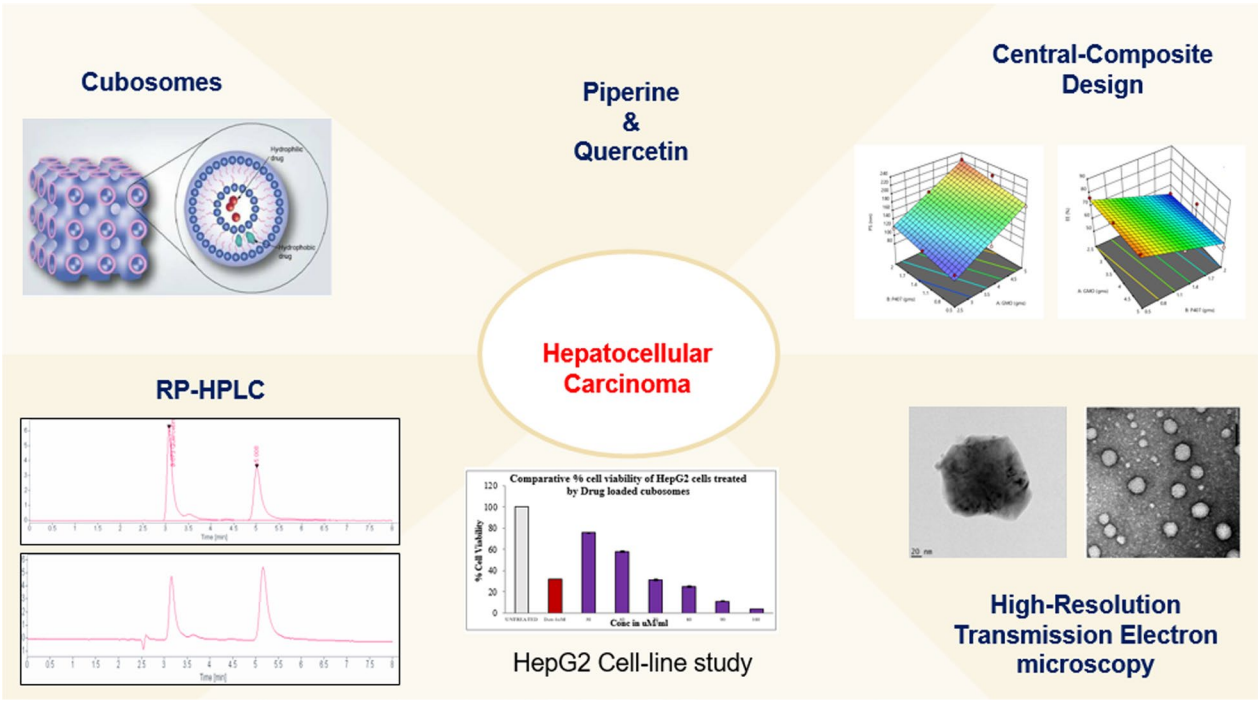
Graphical abstract



Background

In the realm of cancer incidence, primary liver cancer holds the fifth position in terms of frequency, solidifying its status as the second most widespread cancer type [1, 2]. Over the course of several decades, the frequency of cancer cases has consistently risen. This underscores the ongoing significance of cancer as a major public health concern, remaining among the primary contributors to global mortality [3–5]. Despite the emergence of innovative methods like vaccines, immunotherapy, and gene therapy, which exhibit encouraging treatment outcomes for diverse cancer forms, chemotherapy retains its position as a fundamental strategy in the medical management of cancer [6–8].

Natural phytoconstituents play a vital role in impeding diverse mechanisms linked to cancer, including cellular division, cell proliferation, angiogenesis, programmed cell death (apoptosis), and the spread of cancer cells (metastasis) [9, 10]. Numerous herbs and phytoconstituents are utilized for the treatment of chronic liver ailments due to their cost-effectiveness, high safety thresholds, long-lasting positive effects, and minimal adverse reactions [11, 12]. Previous studies suggest that these natural compounds can protect liver cells through diverse mechanisms, including virus elimination, suppression of fibrogenesis, inhibition of oxidative damage, and reduction of carcinogenesis [13–15]. These herbal remedies effectively inhibit cell growth at different stages of the cell cycle, disrupt signaling pathways, and suppress gene expression, leading to the prevention of multiple types of cancer [16–18].

Quercetin, a member of the flavonoid family, possesses notable antioxidant, anti-inflammatory, and immunoregulatory properties, which contribute to its potential in preventing and treating conditions such as cardiovascular diseases, diabetes, neurodegenerative disorders, and cerebrovascular diseases [19–21]. Moreover, studies have reported the inhibitory effects of quercetin on hepatocellular carcinoma (HCC), showing that it can hinder HCC growth by suppressing proliferation and/or promoting apoptosis [22–25]. Numerous pharmacological investigations have indicated the promising capacity of piperine as a viable medication against cancer [26–28]. Its mechanism involves the suppression of pro-survival pathways and the initiation of apoptosis across diverse cancer cell variations. Piperine, identified as a potential anticancer compound capable of regulating autophagy, has demonstrated impressive success in combatting cancer within living organisms [29–31]. Recent research indicates that piperine has been utilized both as a standalone treatment

and in combination with other drugs to enhance their anticancer effects and effectively manage the process of carcinogenesis [32].

Regrettably, these two medications exhibit limited solubility in water and lead to significant problems linked to sensitivity to light. Furthermore, quercetin has a tendency to experience swift chemical deterioration under conditions of acidic gastric pH. Hence, the integration of these two pharmaceutical agents into a well-suited, singularly lipid-mediated nanostructured arrangement, particularly through cubosomes, proves advantageous in surmounting crucial challenges. Cubosomes emerge as nanoparticles containing lipids and surfactants, showcasing an exceptional cubic liquid crystalline configuration [33, 34]. These minute entities, smaller than a micron, display a dual continuous character, boasting a substantial expanse of surface. Comprising amphiphilic components like polar and nonpolar polymers, lipids, and surfactants, cubosomes possess the innate ability to autonomously structure themselves. The genesis of cubosomes hinges on the self-organization of particular surfactant entities [35, 36].

The clinical effectiveness of anticancer drugs is limited because they tend to spread throughout the body without precision, resulting in unintended harm to healthy tissues and consequent side effects [37]. To address this challenge, researchers have introduced diverse drug transporters designed to transport therapeutic substances with precision to particular locations. One of the most successful approaches involves the utilization of nanocarriers, which capitalize on the enhanced permeation and retention phenomenon, allowing them to accurately target the afflicted areas while minimizing impact on unaffected tissues [38, 39].

Consequently, within this current investigation, our contemplation revolves around crafting a cubosome-based nanoformulation that encapsulates quercetin and piperine. The attainment of desired therapeutic effects from any medicinal agent hinges on precise assessments of the entrapped substances within the nanoformulation. Hence, our objective resides in the development of a reverse phase-high-performance liquid chromatography (RP-HPLC) technique, enabling the simultaneous quantification of both quercetin and piperine loaded within the cubosomes.

Methods

Materials

Quercetin (≥95%) was procured from Ausen Chemical Co., Ltd. in China, while piperine (≥97%) was acquired from Sigma-Aldrich India. Glyceryl monooleate (GMO) was kindly provided by Mohini Organics India. Poloxamer was obtained from Sigma-Aldrich in India, while potassium dihydrogen phosphate and sodium hydroxide were sourced from Merck Life Science Pvt. Ltd., India. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) solutions were acquired from Sigma-Aldrich in India. High-performance liquid chromatography (HPLC) grade methanol and various other essential chemicals were obtained from Merck Life Science Pvt. Ltd.

Compatibility studies

To evaluate the compatibility of piperine and quercetin and search for any possible interactions between the two compounds, Fourier transform infrared spectroscopy (FT-IR) was performed [40]. In a nutshell, piperine and quercetin were mixed together in a 1:1 ratio (2 mg), and the mixture was kept at 25 ± 2 °C and 60 ± 5% relative humidity (%RH) for 28 days. Piperine, quercetin, and the mixture underwent FT-IR analysis. The effects of piperine, quercetin, and their combination were studied using FT-IR.

Experimental design

Design-Expert software version 13.0 was used to create the experiments (Stat-Ease Inc., Minneapolis, MN, USA). The program was used to design and produce a total of 11 formulations [41–43]. To optimize the prepared cubosome formulations, a two-factor three-level (3^2) experimental design was employed. The independent variables considered were the amount of glyceryl monooleate (X_1) and Poloxamer 407 (X_2). Meanwhile, the dependent variables assessed were particle size (Y_1) and entrapment efficiency (Y_2). The statistical plan included three different concentrations of Poloxamer 407 (0.5, 1.25, and 2%) and three different concentrations of glyceryl monooleate (2.5, 3.75, and 5%) (Table 1).

Table 1 Coded values of 3^2 full factorial design for formulation of cubosomes

Independent variables	Levels		
	Low (− 1)	Medium (0)	High (+1)
X_1 = GMO	2.5	3.75	5
X_2 = Poloxamer 407	0.5	1.25	2
Independent variables	Constraints	Importance	
X_1 = GMO	In range	+++	
X_2 = Poloxamer 407	In range	+++	
Dependent variables			
Y_1 = Particle size	Minimum	+++	
Y_2 = % EE	Maximum	+++	

Preparation of cubosomes

Piperine- and quercetin-loaded cubosomes were prepared using the top-down method with the aid of a magnetic stirrer and homogenizer. The process began by melting the lipid (2.5 to 5%), followed by dissolving a precisely weighed quantity of the drug (0.1%) in the melted glyceryl monooleate (GMO). This mixture was then transferred into the melted Poloxamer 407 (0.5 to 2%), resulting in the formation of a cubic phase [44–47]. To reach the desired volume (30 mL), a measured amount of water was added dropwise using a syringe. The mixture was homogenized using an Ultraturaxx homogenizer at 12,000 rpm for 15 min and subsequently sonicated for 15 min to obtain cubosomes. The prepared cubosomes were then stored in a cool environment. Various formulations of PQC (piperine–quercetin cubosomes) were meticulously prepared, and their respective compositions are elucidated in Table 2.

Particle size, PDI and entrapment efficiency

The mean vesicle size and polydispersity index (PDI) of cubosomes loaded with piperine and quercetin were analyzed using a zetasizer. For the analysis, 1 mL of the formulation was diluted with 20 mL of milli-Q water. To separate the untrapped drug, samples from each formulation were subjected to centrifugation at 15,000 rpm and 4 °C for a duration of 1 h. The resulting supernatant was collected and then appropriately diluted with methanol. The estimation of the unbound drug was performed using UV–Vis spectroscopy at a wavelength of 273 nm. The percentage of drug entrapment was calculated using the relevant formula [48, 49].

Table 2 CCD experimental runs with obtained response

Formulation code	X_1 (%)	X_2 (%)	Y_1	Y_2
C1	2.5	0.5	102.36	76.43
C2	2.5	1.25	109.75	59.54
C3	2.5	2	118.24	53.28
C4	3.75	0.5	124.44	79.83
C5	3.75	1.25	149.36	61.2
C6	3.75	1.25	151.27	62.3
C7	3.75	1.25	154.2	64.83
C8	3.75	1.25	172.7	65.9
C9	5	0.5	179.6	81.23
C10	5	1.25	216	67.28
C11	5	2	222.4	54.01

X_1 = amount of GMO, X_2 = amount of Poloxamer 407, Y_1 = Particle size, Y_2 = Entrapment efficiency

In vitro release study of cubosomes

The in vitro release study of cubosomes was conducted using the dynamic dialysis method (USP II method). The investigation into drug release employed a tablet dissolution testing apparatus (Electrolab, EDT-08LX) featuring a low-volume conversion kit (EDT-08L/08L × 150 ml). This specialized setup was specifically chosen for assessing the release of the drug from the nanoformulation. Prior to the study, the dialysis membrane was soaked overnight [50–52]. Briefly, PQC nanoformulation (5 ml) was transferred to a dialysis bag, which was immersed into a 100 ml of PBS (pH 5.5) containing tween 80 (0.1%). The temperature of dissolution medium was kept at 37 ± 0.5 °C with a stirring speed of 50 rpm. At predetermined time intervals (0.5, 1, 2, 3, 4, 6, 12 and 24 h), aliquots of 1 mL were withdrawn for analysis. The withdrawn aliquots of the drug release medium were replaced with same volume of fresh buffer to maintain the sink condition. The drug concentrations in the samples were analyzed using spectrophotometry at the maximum wavelength (λ max) of 273 nm.

Transmission electron microscopy (TEM)

The surface morphology and structure of optimized cubosomes were examined using a high-resolution transmission electron microscope (JEM-2100, Jeol, Tokyo, Japan). A small volume of the optimized cubosomes was applied onto a carbon-coated grid, allowing the sample to adhere. Subsequently, the specimen was stained with uranyl acetate for a duration of 5 min, followed by staining with lead citrate for 2 min. The grid containing the stained sample was then observed using a transmission electron microscope, with an appropriate level of magnification (20 nm and 100 nm) chosen to capture images [53, 54].

High-performance liquid chromatography (HPLC)

The Agilent Technologies 1220 Infinity II HPLC system was used for quantification of piperine and quercetin in optimized cubosomal formulation. Utilizing a reversed-phase C-18 column (5 µm, 250 × 4.6 mm, ZORBAX), chromatographic separation was accomplished [55–58]. After conducting numerous trials, the chromatographic conditions were fine-tuned by considering factors such as peak area, retention time, tailing factor, and theoretical plates. The optimal mobile phase was composed of methanol and 0.1% formic acid in water, in the ratio of 80:20. Samples were analyzed at the flow rate of 1 mL/min, and the detection wavelength was set at 273 nm. The prepared cubosomes were centrifuged for 30 min at 15,000 rpm. The supernatant (0.1 mL) was pipetted using a micropipette and made up the volume up to 10 mL of volumetric flask using mobile phase. For each analysis,

a 10 µl sample was injected into the column. According to ICH Q2 (R1) criteria, the developed HPLC method's validation was carried out. Linearity, LOD, LOQ, precision, accuracy and robustness were all used to thoroughly evaluate the optimized method.

Cytotoxicity analysis

For this study, the HepG2 cell line, derived from human hepatocellular adenocarcinoma, was procured from NCCS in Pune, India. The cells were cultivated in DMEM-high glucose medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic–antimycotic solution [59, 60]. Maintaining optimal conditions, the cells were cultured in a controlled environment with 5% CO_2, 18–20% O_2 and a temperature of 37 °C within a CO_2 incubator. Sub-culturing of the cells was carried out every 2 days, and passage number 34 was employed for the experimental procedures. Transfer 200 µl of cell suspension into individual wells of a 96-well plate, targeting a cell density of 20,000 cells per well. Avoid introducing the test agent at this stage. Allow the cells to proliferate for approximately 24 h. Distinct concentrations of cubosomal solutions (50, 60, 70, 80, 90, and 100 µg/ml) were prepared in a solution containing 0.1% DMSO. Subsequently, 50 µl of each concentration's solution was individually introduced into designated wells containing the cells. The plate was then placed in an incubator set at 37 °C, 5% CO_2, and 90% humidity for a 24-h duration. A control group was processed without the addition of this formulation. Following the incubation period, 15 µl of MTT solution (5 mg/ml in PBS) was added to the wells, and the plate was further incubated for 4 h at 37 °C. Post-incubation, 100 µl of DMSO was introduced to dissolve the formazan crystals that had formed. The absorbance of the cells was measured at 570 nm utilizing an ELISA plate reader. The mean values for cell viability were compared against the control to assess the impact of cubosomes on the cells. A graphical representation was plotted, correlating cell viability (%) against the concentration levels of the formulation.

Statistical optimization

The experiments conducted in this study were replicated in triplicate, and the outcomes were reported as the mean accompanied by the standard deviation. Both ANOVA and Student's *t* test were executed, determining statistical significance for results at a threshold of $p < 0.05$.

Results

Compatibility studies

The Fourier transform infrared (FTIR) analysis of piperine and quercetin, as depicted in Fig. 1, unveiled the presence of various discernible functional groups. Notably, the spectrum exhibited distinct peaks corresponding to specific vibrational modes, including the aromatic C-H stretch at 2922.61 cm^{-1}, the alcohol O–H stretch at 3393.02 cm^{-1}, the C–O stretch at 1246.50 cm^{-1}, and the aromatic C–C stretch at 1640.32 cm^{-1}. Intriguingly, the physical mixture of the drug and excipients manifested the persistence of these characteristic peaks, underscoring the compatibility of the components. This observation serves to corroborate that the unique chemical identities of both the drug and the excipients remain intact in the composite system.

Experimental design

Based on the regression coefficient determined in the ANOVA study, it was discovered that independent variables X_1 (GMO) and X_2 (Poloxamer-407) have a positive sign, indicating a synergistic influence on the outcome Y_1 (PS). It was discovered through graphical presentation, namely the response surface plot that raising the level of variable X_1 and X_2 tends to raise the value of response Y_1 (Fig. 2). Both independent variables X_1 and X_2 had a negative sign, indicating an antagonistic influence on the response Y_2. Graphical evidence revealed that as the level of both variables, X_1 and X_2, is increased, the value of response Y_2 was dropped (Fig. 3). Utilizing the Design-Expert software by Stat-Ease, Inc. (version 13.0), it was determined that all responses yielded satisfactory outcomes. The shaded zone with yellow color in the design space (Overlay plot) depicted in Fig. 4 represents the region of successful operating ranges. Design space facilitated the identification of optimal values for the independent parameters: GMO (2.5%) and Poloxamer (0.5%) under these specific concentrations. The model's exceptional ability to forecast values for the response variables was showcased by the remarkably low prediction error (5%) observed between the predicted values and the actual observations.

Particle size, PDI and entrapment efficiency

The drug-loaded cubosome nanoformulation was prepared through homogenization method, resulting in mean particle sizes of 102.36 nm and 222.4 nm, respectively. The cubosomes exhibited an average PDI in value range 0.1831 to 0.8894. These PDI values are indicative of a narrow and homogeneous particle size distribution, as they are less than 0.9. The zeta potential is a significant variable for evaluating the stability of nanoparticulate systems. Zeta potential was recorded to be between − 13.6 and − 38.9 mV. These zeta potential readings suggest that the cubosome nanoparticles are moderately stable. The presence of free fatty acids within glycerol monooleate's lipid structure, which imparts this negative charge, is the cause of the negative charge that was detected.

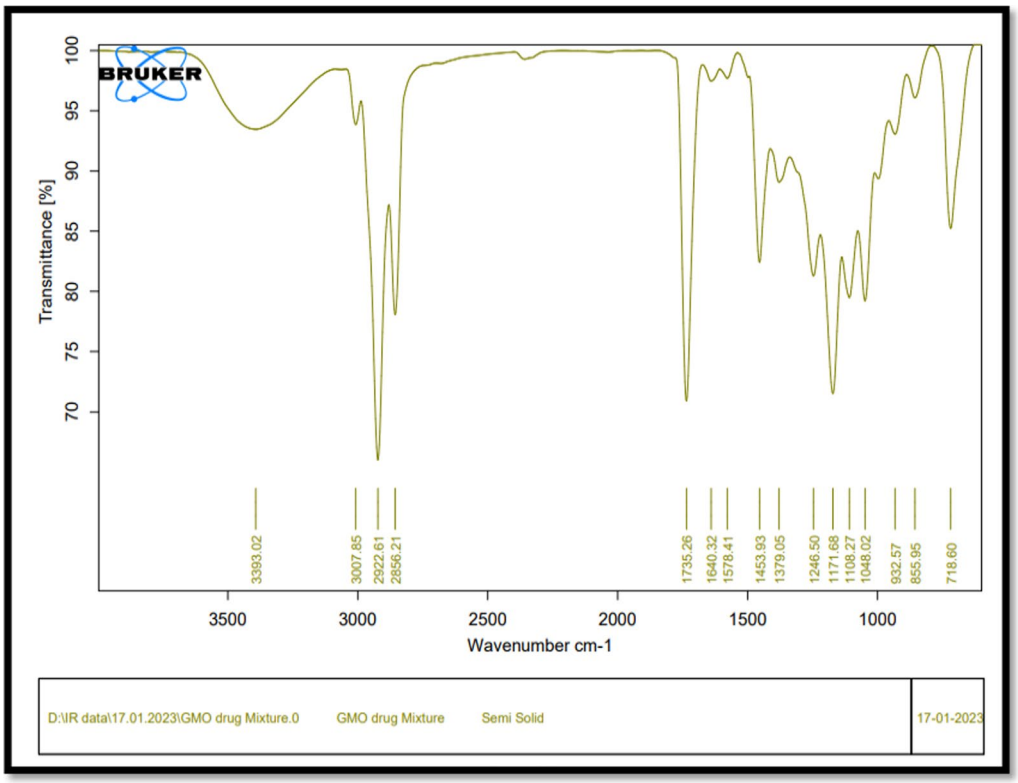


Fig. 1 FTIR spectrum of physical mixture (GMO, Poloxamer 407, piperine, and quercetin)

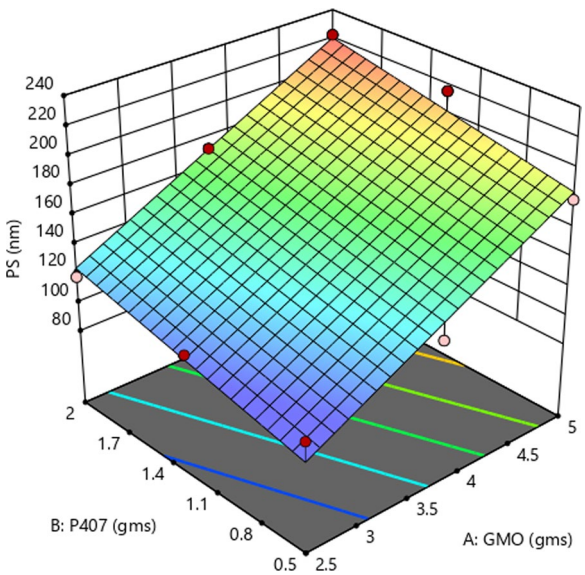


Fig. 2 Response surface plot for response Y_1 (Particle size)

Our analysis of the percentage of free and total drug in the cubosome nanoformulation revealed that the encapsulation efficiency (EE) for piperine and quercetin exhibited a range of 53.28% to 81.23%. The optimized

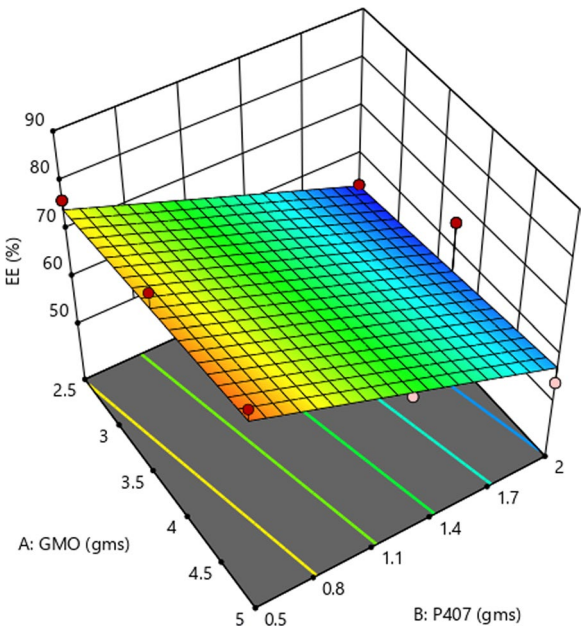


Fig. 3 Response surface plot for response Y_2 (Entrapment efficiency)

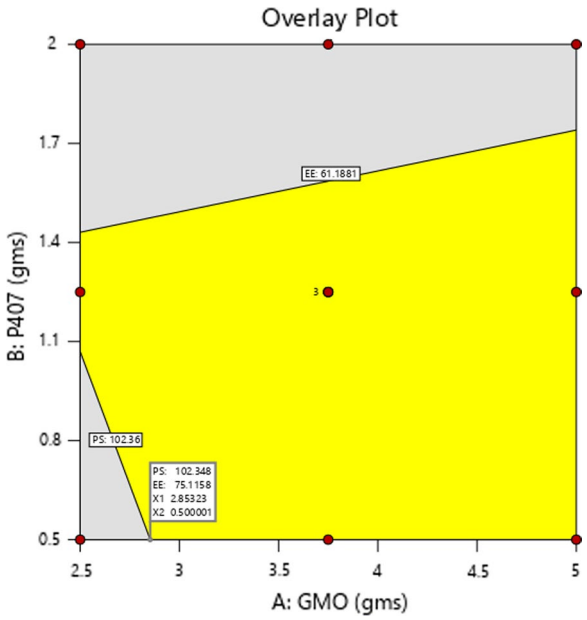


Fig. 4 Design space overlay plot

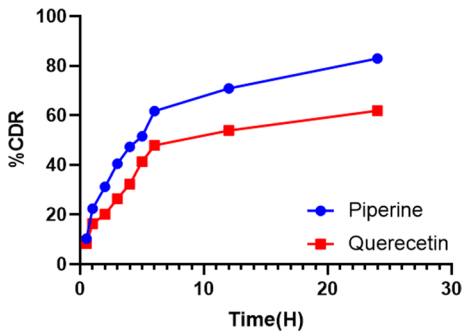


Fig. 5 Cumulative drug release of PQC formulation

concentration of surfactants used is probably responsible for the cubosomes substantially higher EE percentages. Significant amounts of quercetin and piperine were successfully solubilized by these surfactants within the lipid vesicles. The amounts of these substances were thereby markedly increased within the cubosome formulation.

In vitro release study of cubosomes

The in vitro drug release profiles of piperine and quercetin from cubosomes are meticulously illustrated in Fig. 5. The release study, specifically focusing on the optimized batch of the PQC nanoformulation (designated as PQC1), was conducted in a buffer with a pH of 5.5. This investigation was carried out utilizing a tablet dissolution testing apparatus equipped with a low-volume conversion kit. Broadly, the bicontinuous cubosome nanoformulations exhibited a biphasic burst release characterized by a diffusion-based mechanism emanating from the cubic-phase matrix structure. The release of both piperine and quercetin from the cubosome formulation displayed a biphasic pattern. In the initial hour, there was a notable burst release, accounting for approximately 22.43% of piperine and 16.40% of quercetin. Subsequently, after a 24-h duration, the cumulative release escalated to 82.92% for piperine and 61.04% for quercetin.

Transmission electron microscopy (TEM)

The surface morphology and structural integrity of the optimized PQC nanoformulation (PQC1) were meticulously scrutinized and substantiated through high-resolution transmission electron microscopy (HR-TEM) imaging. The resulting images revealed that the cubosome particles exhibited a distinctly cubic shape, characterized by a uniform and smooth surface with minimal curvature, as depicted in Fig. 6A, B. Notably, the

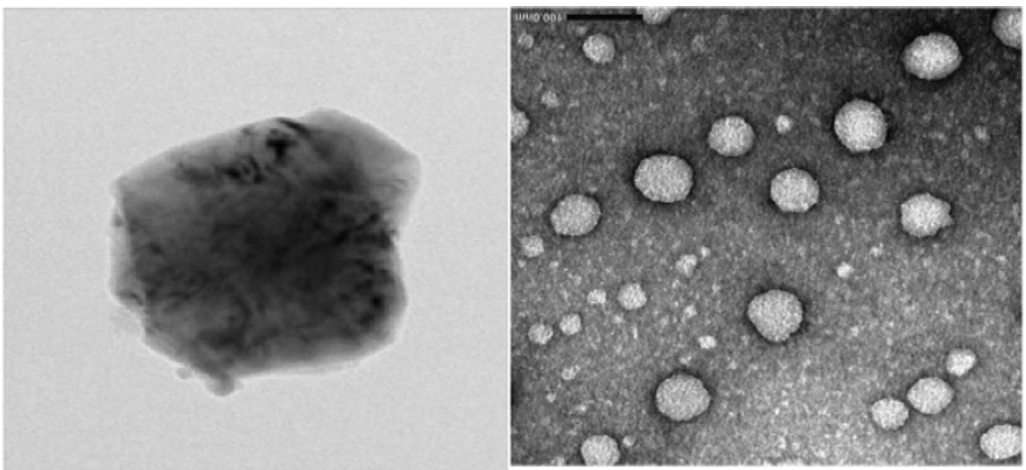


Fig. 6 HR-TEM images of optimized cubosomal nanoformulation (PQC1)

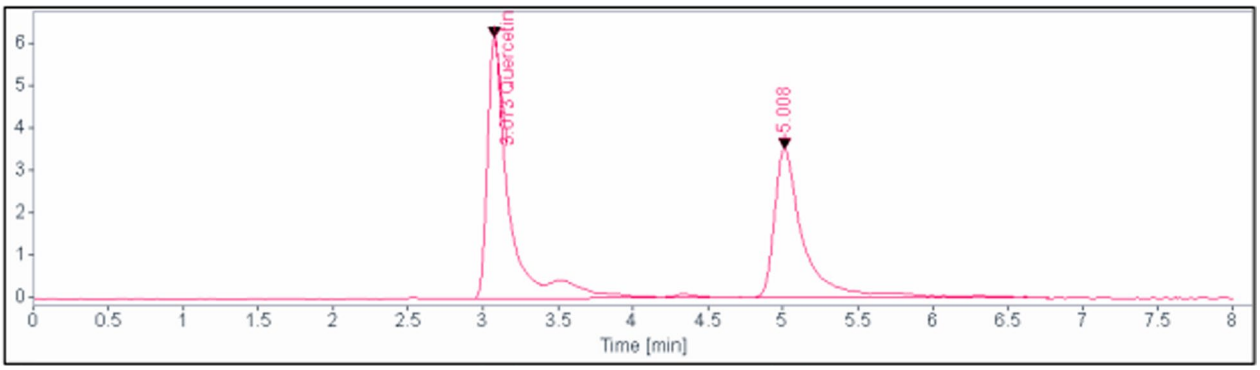


Fig. 7 HPLC chromatogram depicting standard quercetin and piperine

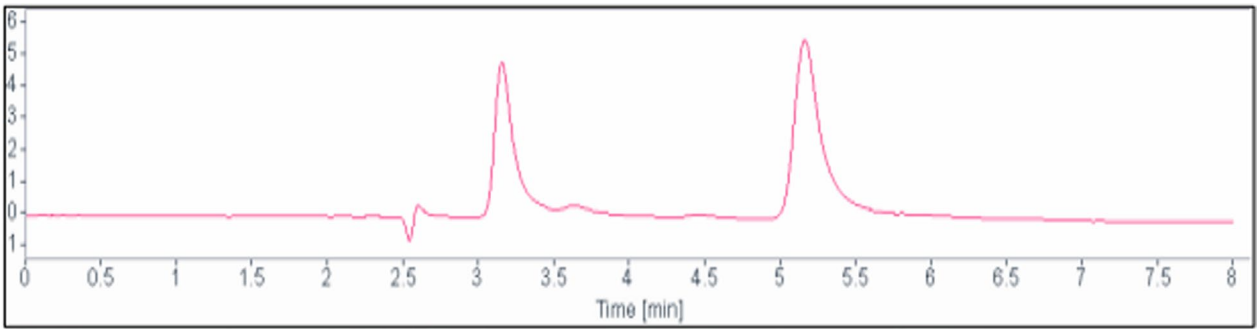


Fig. 8 HPLC chromatogram depicting quercetin and piperine in cubosomes

particles displayed a scattering pattern indicative of their nanoscale dimensions, further accentuating their finely tuned and compact structure.

High-performance liquid chromatography (HPLC)

The optimized method has proven its efficacy in the determination of formulated cubosomes, revealing distinct peaks for both quercetin and piperine. Notably, these peaks were precisely measured at 3.055 and 5.067 min, respectively. The consistent retention times underscore the robustness of the separation method, offering compelling evidence for the successful application of this approach in isolating the two pharmaceuticals within the cubosomes, as illustrated in Figs. 7 and 8. To ensure that the optimized HPLC method is appropriate for the intended use as outlined in ICH Q2 (R1) standards, it has undergone validation. Table 3 provides a summary of the proposed HPLC method's validation parameters, which were determined to be within the ICH Guidelines' standard limits.

Table 3 Summary of validation parameter

S. no.	Validation parameters	Quercetin	Piperine
1	Linearity		
	Linearity range (µg/mL)	2–10	2–10
	Correlation-coefficient	< 0.999	< 0.998
2	LOD (µg/mL)	0.32	0.41
	LOQ(µg/mL)	0.98	1.32
3	Precision		
	Intra-day (%RSD)	0.521 ± 0.07	0.2 ± 0.003
	Inter-day (%RSD)	1.25 ± 0.027	0.42 ± 0.01
4	Robustness		
	Change in Mobile phase volume (%RSD)	0.710 ± 0.047	0.28 ± 0.041
	Change in flow rate (%RSD)	0.323 ± 0.02	0.83 ± 0.018
5	Accuracy		
	50% recovery	98.75 ± 0.02	101.31 ± 0.24
	100% recovery	100.24 ± 0.37	100.78 ± 0.51
	150% recovery	98.21 ± 0.52	99.21 ± 0.23

Cytotoxicity analysis

The statistical findings from the MTT cell cytotoxicity study revealed that the examined compounds exhibited noteworthy cytotoxic attributes against HepG2 cells. Following a 24-h incubation period, the % cell viability of cubosomes at a concentration of 100 µM/mL was determined to be 4.05%. A standard control utilizing 1 µM/mL of doxorubicin was employed, and the cell viability for the

standard was established at 32.25% (Fig. 9). The determination of the IC50 value for the cubosomes, based on the percentage drug loading, unequivocally indicates that the designed nanoformulation possesses a superior anticancer effect compared to the standard drug. This superiority may be attributed to its nanosized dimensions, sustained release properties and targeted delivery to cancer cells.

Discussion

This study takes a step toward enhancing liver cancer treatment by focusing on cubosomal formulations containing piperine and quercetin. Piperine and quercetin are compounds known for their potential anticancer properties. By formulating these compounds into cubosomes, the aim is to augment their activity against hepatocellular carcinoma. To assess the compatibility of piperine and quercetin and explore potential interactions between these two compounds, Fourier transform infrared spectroscopy (FT-IR) was conducted. The obtained spectrum revealed clear peaks associated with specific vibrational modes, such as the aromatic C–H stretch observed at 2922.61 cm^{-1}, the alcohol O–H stretch at 3393.02 cm^{-1}, the C–O stretch at 1246.50 cm^{-1}, and the aromatic C–C stretch at 1640.32 cm^{-1}. This observation serves to corroborate that the unique chemical identities of both the drug and the excipients remain intact in the composite system.

The experimental design of this study utilized a central-composite design, guided by the Design-Expert® software. Key formulation variables were the concentration of glyceryl monooleate (GMO) and Poloxamer-407, while particle size (PS) and entrapment efficiency (EE %) were chosen as dependent responses. This approach provides a systematic and statistical method to optimize the formulation for the desired properties. The optimized cubosomal formulation, with a composition of 2.5% (w/w) GMO and 0.5% (w/w) Poloxamer 407, was validated using various techniques. The predicted values for PS and EE% were 102.34 and 76.43%, respectively, indicating successful optimization based on the experimental design. The optimized nanoformulation of piperine and quercetin (PQC1) underwent thorough examination of its surface morphology and structural integrity using high-resolution transmission electron microscopy (HR-TEM) imaging. The images obtained depicted cubosome particles with a well-defined cubic shape, showcasing a consistently smooth and uniform surface with minimal curvature.

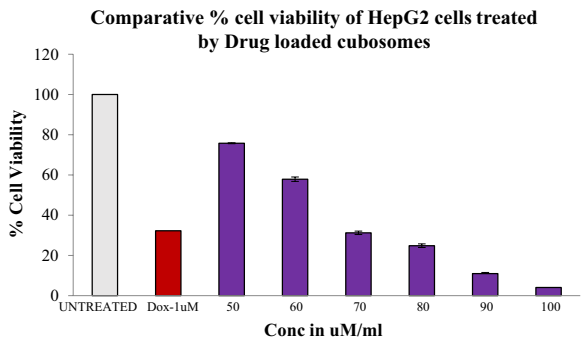


Fig. 9 The overlaid bar graph depicting the percentage of cell viability for HepG2 cell lines

The in vitro release study, specifically focusing on the optimized batch of the PQC nanoformulation (designated as PQC1), was conducted in a buffer with a pH of 4.5. After a 24-h duration, the cumulative release escalated to 82.92% for piperine and 61.04% for quercetin. High-performance liquid chromatography was utilized to quantify the concentration of piperine and quercetin within the optimized cubosomal nanoparticles. Significantly, the distinct peaks for piperine and quercetin were precisely identified at 3.055 and 5.067 min, respectively. The consistent retention times emphasize the reliability of the separation method, providing compelling evidence for the effective application of this approach in isolating the two pharmaceuticals within the cubosomes. The cytotoxicity assessment revealed that the optimized cubosomal formulation exhibited enhanced efficacy on the HepG2 cancer cell line, even at lower concentrations compared to the standard treatment. This superior cytotoxic effect specifically on the liver cancer cell line suggests the potential of cubosomes as an effective carrier for delivering piperine and quercetin to address hepatocellular carcinoma.

The findings of this study highlight the promise of cubosomes in liver cancer therapy. The targeted delivery system not only optimizes drug efficacy but also minimizes side effects, addressing a critical need in the complex landscape of liver cancer treatment. The use of advanced techniques such as HR-TEM and high-performance liquid chromatography adds credibility to the study, ensuring a thorough validation of the optimized cubosomal formulation. In conclusion, this research contributes to the growing body of knowledge seeking innovative solutions for liver cancer treatment. The effectiveness of cubosomal formulations containing piperine and quercetin against hepatocellular carcinoma suggests a potential breakthrough in precision therapy. As further research and clinical trials unfold, these findings may pave the way for a more targeted and efficient approach to combat liver cancer, ultimately improving patient outcomes on a global scale.

Conclusion

The objective of this research was to develop precision-targeted cubosomes containing piperine and quercetin to address Hepatocellular carcinoma. Effective formulation and fine-tuning of cubosomes loaded with piperine and quercetin were accomplished employing central composite factorial design methodology in conjunction with the Design-expert software. The prepared cubosomes demonstrated well-suited nanoscale vesicles, with particle dimensions falling within the nanoscale and achieving peak entrapment efficiency levels. Following the design of experiments (DoE) and the desirability criteria, it was determined that formulation (C1) containing GMO (2.5%) and Poloxamer 407 (0.5%) stood out as the most favorable selection, exhibiting the most compact particle size (102.3 nm) and greatest entrapment efficiency (76.43%). An analytical technique was established for piperine and quercetin using HPLC under standard environmental conditions. The solvent system utilized consisted of HPLC-grade methanol and 0.1% formic acid in water an 80:20 ratio. The quantitative analysis demonstrated well characteristics peaks for both drugs in the optimized cubosomal formulation, with a retention time of 3.0 min for quercetin and 5.0 min for piperine. The investigation on the HepG2 cell line using the cubosomes loaded with piperine and quercetin, carried out through the MTT assay. Following a 24-h incubation period, the % cell viability of cubosomes at a concentration of 100 µM/mL was determined to be 4.05%. A standard control utilizing 1 µM/mL of doxorubicin was employed, and the cell viability for the standard was established at 32.25%. This outcome serves as confirmation of the formulation's effectiveness in treating hepatocellular carcinoma; this formulation stands as a promising and preferred dosage form poised to be a leading option in the near future.

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Author contributions
We affirm that "all authors have read and approved the manuscript." Each contributor played an equal role and actively engaged in this research project. Both PB and RK conscientiously reviewed the manuscript titled "Dual Drug-Loaded Cubosome Nanoparticles for Hepatocellular Carcinoma: A Design of Experiment Approach for Optimization and In Vitro Evaluation." Their efforts were conducted under the supervision of Dr. VSM, who offered guidance and assistance in navigating research complexities.

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Availability of data and materials
The research work has been carried out by us, and we assure you that it can be provided to you whenever required.

Declarations

Ethics approval and consent to participate
No.

Consent for publication
No.

Competing interests
The authors declare that they have no competing interests.

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