In vitro and in vivo evaluation of cubosomes containing 5-fluorouracil for liver targeting

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Abstract  The objective of this study was to prepare cubosomal nanoparticles containing a hydrophilic anticancer drug 5-fluorouracil (5-FU) for liver targeting. Cubosomal dispersions were prepared by disrupting a cubic gel phase of monoolein and water in the presence of Poloxamer 407 as a stabilizer. Cubosomes loaded with 5-FU were characterized in vitro and in vivo. In vitro, 5-FU-loaded cubosomes entrapped 31.21\% drug and revealed nanometer-sized particles with a narrow particle size distribution. In vitro 5-FU release from cubosomes exhibited a phase of rapid release of about half of the entrapped drug during the first hour, followed by a relatively slower drug release as compared to 5-FU solution. In vivo biodistribution experiments indicated that the cubosomal formulation significantly ($P<0.05$) increased 5-FU liver concentration, a value approximately 5-fold greater than that observed with a 5-FU solution. However, serum serological results and histopathological findings revealed greater hepatocellular damage in rats treated with cubosomal formulation. These results demonstrate the successful development of cubosomal nanoparticles containing 5-FU for liver targeting. However, further studies are required to evaluate hepatotoxicity and in vivo antitumor activity of lower doses of 5-FU cubosomal formulation in treatment of liver cancer.

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1. Introduction

5-Fluorouracil (5-FU), a water-soluble fluorinated pyrimidine analog, is an antineoplastic agent which is widely used alone or in combination chemotherapy regimens for the treatment of advanced gastrointestinal cancers including hepatocellular carcinoma. However, the clinical use of 5-FU is limited by its gastrointestinal toxicity, hematologic side effects and severe bone marrow disturbances. Moreover, because of the short plasma half-life (10–20 min) and the high rate of metabolism of this drug in the body, the maintenance of a therapeutic serum concentration requires the continuous administration of high doses. Elevated plasma levels of 5-FU can cause severe side effects and the antitumor effects of this drug depend on exposure duration rather than plasma concentration. Previous reports indicated that sustained release formulations of 5-FU and selective delivery to the tumor site not only improve the antitumor activity but also reduce side effects of 5-FU as compared with the clinically-available 5-FU formulation.

Glycerol monooleate (GMO) is known to spontaneously form liquid crystalline cubic phases in excess water, consisting of bicontinuous lipid bilayers extending in three dimensions, separating two networks of water channels. Due to GMO's unique structure, cubic phases are able to incorporate and control the release of drugs of various molecular weights and polarities. Three macroscopic forms of cubic phase are typically encountered: precursor, bulk and particulate (i.e., cubosomes). Precursor materials are usually liquid and form cubic phase only in response to some stimulus, like dilution. Bulk forms of cubic phase are viscous liquid crystalline materials, usually hydrated monoolein, often with a drug incorporated in their structure. The high viscosity, biodegradability, ability to incorporate and deliver drugs of varying sizes and water solubilities and the ability to enhance the chemical and/or physical stability of the incorporated drugs make the bulk cubic gel an excellent candidate for use as a drug delivery matrix. However, the high viscosity and stiffness of the cubic gel limit its potential use as the delivery system by itself.

The emulsification of the cubic lipid phases in water results in the production of cubosomes that can be defined as nanoparticulate dispersions systems characterized by high biocompatibility and bioadhesivity. It has been demonstrated that the dispersed particles retain the internal structure of the bulk phase and its properties. Because of their properties, these versatile delivery systems can be administered by different routes (i.e., orally, parenterally or percutaneously). In comparison with the bulk cubic gel, cubosomal dispersions present some advantages, such as a larger surface area and high fluidity (low viscosity). However, due to their extremely small size (and the resulting short diffusion pathways) cubosomes are unlikely to offer similar opportunities to control drug release as bulk cubic phases do. In addition, the large amount of water present during cubosome formation renders the incorporation of water-soluble drugs difficult.

This work describes a simple method for preparation of cubic phase gel matrix containing small molecular weight hydrophilic drug (5-FU) that could be dispersed with water to form a cubosomal nanoparticle dispersion prior to subcutaneous administration. The 5-FU-loaded cubosomes were evaluated for their in vitro and in vivo characteristics in an attempt to explore their potential as a targeted drug delivery system that could provide a maximum concentration of 5-FU in the liver tissues. This is expected to improve the efficacy of low doses of the drug and to minimize the side effects associated with the higher doses of 5-FU when used in treatment of hepatocellular carcinoma.

2. Materials and methods

2.1. Materials

Myverol® 18–99 K, as a source of monoolein, was a gift from Kerry Ingredients & Flavours (Zwijndrecht, Netherlands). Poloxamer 407 and 5-FU were purchased from Sigma-Aldrich Chemical Company (Milwaukee, USA). Milli-Q purified water was used for all experiments. Other reagents were of analytical grade.

2.2. Preparation of blank and 5-FU-loaded cubic gel

For blank cubic gel, GMO (2.25 g) and poloxamer 407 (0.25 g) were melted at 70 °C in a water bath. The obtained molten solution was added dropwise to 4 mL of deionized water (70 °C) and vortex mixed at high speed at room temperature to achieve homogeneous state. The mixture was equilibrated at room temperature for 48 h to obtain the cubic gel. The drug-loaded cubic gel was prepared by dissolving 50 mg of 5-FU in 4 mL deionized water before addition of the GMO/poloxamer 407 molten solution. The remaining process followed the same steps as described for preparation of blank cubic gel. The cubic gels were stored at ambient temperature until required.

2.3. Preparation of cubosomal nanoparticle dispersions

To prepare the cubosomal dispersion, the cubic gel was dispersed with 18.50 mL deionized water by vortex at high speed for 3 min. The final concentration of lipid in the dispersion is 10% (w/v) with respect to the final dispersion weight. The final 5-FU concentration in cubosomal dispersion was 2 mg/g cubosomal dispersion.

2.4. Characterization of cubosomes

2.4.1. Morphology of cubosomes

Morphological examination of cubosomal nanoparticles was carried out using a transmission electron microscope (FEI, The Netherlands), modal: Tecnai G20 equipped with super twin lens, a Lab6 electron source and operated at 60 kV. A droplet of cubosomes dispersion was placed on a 200 mesh carbon-coated copper grid, and the excess fluid was removed by an absorbent filter paper. The samples were stained with 1% sodium phosphotungstate solution and were viewed using magnification up to 1,000,000 x.

2.4.2. Particle size analysis

The particle size distribution (Z-average) and polydispersity index (PDI) of cubosomal dispersions were determined by dynamic light scattering using Zeta Sizer Nano-series (Nano ZS, Malvern, Worcestershire, UK). Samples were diluted (100-fold) with deionized water and measured at 25 ± 0.5 °C in triplicate.

2.4.3. Entrapment efficiency

The drug entrapment efficiency was determined by ultrafiltration centrifugation. 1 mL of freshly prepared 5-FU loaded cubosomal dispersion was diluted to 10 mL with deionized water and 3 mL of the diluted samples was placed in centrifuge tubes (Amicon Ultra 3000 MWCO, Millipore, USA) and centrifuged at 4000 rpm for 15 min. As some drugs are adsorbed to the ultrafiltration membrane to a certain extent, the drug adsorption to the ultrafiltration membrane was investigated by filtration of simple drug solution of
known concentrations through the membrane and measuring drug concentrations in the ultrafiltrate. Free 5-FU contained in filtrate was measured spectrophotometrically at $\lambda_{\text{max}} = 266$ nm. The amount of entrapped 5-FU was obtained by subtracting the amount of free drug from the total drug incorporated in 1 mL cubosomal dispersion. The total amount of 5-FU incorporated in 1 mL cubosomal dispersion was determined after addition of 9.0 mL methanol to dissolve the drug loaded-cubosomes. The resultant solution was assayed for the total 5-FU content spectrophotometrically using methanol as blank. The entrapment efficiency (EE) was calculated as follows:

$$\text{EE} (\%) = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug}} \times 100$$

2.4.4. Differential scanning calorimetry (DSC)
To detect any possible change in the physical state of 5-FU entrapped in the cubic gel, DSC was performed on 5-FU-loaded cubic gel, blank cubic gel, pure 5-FU powder, GMO and poloxamer 407 using a thermal analysis system (DSC-60, Shimadzu, Japan). The samples (5 mg) were heated at a constant rate of 10 °C/min in an aluminum pan under a nitrogen atmosphere. A similar empty pan was used as the reference.

2.4.5. X-ray diffraction patterns of the prepared cubic gels as well as pure 5-FU, GMO and poloxamer 407 samples were obtained using the X-ray diffractometer (X’Pert-PRO Diffractometer, PANalytical, Netherlands) with Cu as tube anode. The diffractograms were recorded under the following conditions: the voltage 45 kV, the current 30 mA, the steps 0.02° and the counting rate 0.5 s/step at room temperature. Data were collected using scattering angle $(2\theta)$ ranged 4–50°.

2.4.6. In vitro drug release from cubosomes
In vitro release of 5-FU from cubosomes was evaluated using a dynamic dialysis method. The release rate of drug was determined after separation of free drug from drug-loaded cubosomes by placing the cubosomal dispersion in dialysis tubing (10,000 MWCO, Millipore, Boston, USA) and exhaustively dialyzed for 15 min for several times, each time against 100 mL of phosphate buffer (pH 7.4). The dialysis of free 5-FU was completed after 1 h after which no further drug could be detected in the solution. The dialyzed suspension containing 5-FU-loaded cubosomes (equivalent to 1 mg drug) or plain drug aqueous solution was sealed in a dialysis bag (10,000 MWCO, Millipore, Boston, USA). The dialysis bag was then immersed in 100 mL of phosphate buffer (pH 7.4) thermostatically maintained at 37 ± 0.5 °C and magnetically stirred at 50 rpm. The samples (3 mL) were withdrawn at various time intervals and analyzed by a UV spectrophotometer at 266 nm. Volumes lost by sample withdrawal were replaced with fresh medium. The experiments were conducted in triplicate.

2.5. Stability study
The stability study was carried out on cubic gel containing 5-FU. Samples of cubic gel were stored in tightly closed amber colored glass vials sealed with aluminum foil at refrigeration temperature 4–8 °C for a period of 3 months. The samples were withdrawn at the end of the study period and were dispersed in deionized water by vortex for 3 min. The prepared cubosomal dispersion was subjected for mean particle size and EE (%) measurements. All reported particle size and EE (%) data are the mean of three separate measurements.

2.6. In vitro cytotoxicity of 5-FU-loaded cubosomes
Samples were supplied to the Bioassay-Cell Culture Laboratory (National Research Centre, Cairo, Egypt) to determine the in vitro cytotoxicity of 5-FU cubosomal dispersion (containing free drug and 5-FU-loaded cubosomes) compared to 5-FU solution.

The in vitro cytotoxicity was performed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan. MTT cell viability assay was carried out with human hepatoma HepG2 cell line. Cells were suspended in RPMI 1640 medium containing 1% antibiotic–antimycotic mixture (10,000 U/mL penicillin, 10,000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B), 1% l-glutamine and 10% fetal bovine serum and kept at 37 °C under 5% CO₂. Cells were batch cultured for 10 days, then seeded at concentration of 1 × 10⁵ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO₂ using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (control) or with different concentrations of samples to give a final concentration (100, 50, 25 and 12.5 μg/mL) of 5-FU. After 48 h of incubation, medium was aspirated; 40 μL of MTT salt (2.5 μg/mL) was added to each well and incubated for further 4 h at 37 °C under 5% CO₂. To stop the reaction and dissolving the formed crystals, 200 μL of 10% sodium dodecyl sulfate in deionized water was added to each well and incubated overnight at 37 °C. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, USA) at 595 nm and a reference wavelength of 620 nm. Cell viability was calculated as the percentage of absorbance in wells with the treated cells to that of control cells. A probit analysis was carried for IC₅₀ (the concentration that inhibited cell growth by 50%) determination using SPSS 11 program.

2.7. In vivo evaluation of 5-FU-loaded cubosomes
The protocol of the in vivo studies was approved by the Animal Ethics Committee of Faculty of Pharmacy, Helwan University. The study was conducted in accordance with EC Directive 86/609/EEC for animal experiments.

2.7.1. Biodistribution of 5-FU in rat liver
Eighteen adult male Wistar rats weighing 160–180 g were used in the study. All rats were housed and received similar diet. The rats were divided randomly into 2 groups; each was of 9 rats and all rats were fasted overnight for 12 h with free access to water. On the day of experiment, each rat in group 1 received a single subcutaneous dose of 10 mg/kg of plain 5-FU solution in phosphate-buffered saline and rats in group 2 received the same equivalent doses of 5-FU cubosomal dispersion containing both 5-FU-loaded cubosomes and free 5-FU. The use of cubosomal dispersion was the possible practical solution to attain the required dose of 5-FU in a suitable volume for subcutaneous administration.
After 1, 2 and 3 h of dosing, 3 rats from each group were sacrificed by cervical dislocation. The rats were dissected, and their livers were removed and rinsed with saline solution to remove any adhered debris, blotted dry with filter paper. 1 g of liver tissue was homogenized (Yellow-Line disperser, IKA® Works, Inc., USA) in 3 mL phosphate buffer saline (pH 7.4). The homogenate was centrifuged at 6000 rpm at 4 °C for 30 min to obtain supernatant. The supernatant of liver homogenate was kept frozen until analysis.

2.7.2. Analysis of 5-FU concentration
5-FU concentration in the supernatant of liver homogenate was quantified by a reported liquid chromatography–tandem mass spectrometry (LC-MS/MS) method\(^1\) with slight modifications. Samples (500 μL) was mixed with 50 μL of ammonia and extracted with 6 mL of ethyl acetate. After centrifugation at 5000 rpm for 5 min, 3 mL of the organic layer was evaporated to dryness. The residue was redissolved in 250 μL of the mobile phase and the obtained solution was filtered and a volume of 10 μL filtrate was injected into the LC-MS/MS system.

The LC system consisted of a Thermo Fisher Scientific (San José, USA) Accela HPLC 1200 LC-10AD pumping system, coupled with an Accela autosampler and a Hypersil Gold C18 column (50 mm × 2.0 mm, 2.1 μm, Phenomenex) preceded by a Gemini C18 (4 mm × 3 mm, 5 μm) security guard cartridge (Phenomenex). Separation and elution were achieved using acetonitrile: 0.1% formic acid (90:10, v/v) as the mobile phase at a flow-rate of 0.25 mL/min for a run time of 2 min. Mass spectrometric analysis is carried out using a TSQ Quantum Access AX triple quadrupole mass spectrometer. Data acquisition for quantitation and confirmation is performed in Full scan mode. Samples are individually tuned for each target analyte by direct injection of the individual solution (1 mg/mL). The following working conditions were applied: ionization mode: Heated Electrospray (HESI); polarity: positive ion mode; spray voltage: 300 V; vaporizer and capillary temperature at 400 and 370 °C, respectively; sheath and auxiliary gas pressure at 25 and 5 arbitrary units, respectively; cycle time: 0.7 s. Peak width: full width of a peak at half its maximum height (FWHM) of 0.70 Da. The lower limit of quantification was 0.1 μg/mL. The standard calibration curve for 5-FU was linear (correlation coefficients were >0.9997) over the studied concentration range (0.4–10 μg/mL). Instrument control, data acquisition and data evaluation were performed using Thermo Scientific Xcalibur 2.1 software.

2.7.3. Liver function and histopathological examination
The main objective of this experiment was to evaluate the effect of 5-FU cubosomal formulation on rat’s liver function parameters and the possible histopathological change in liver tissues compared to free 5-FU solution. For this study, 12 male Wistar rats weighing 175–190 g were divided into 4 groups each containing 3 rats. The rats in groups 1 and 2 were treated subcutaneously with 10 mg/kg of free 5-FU aqueous solution and the same equivalent dose of 5-FU cubosomal dispersion for 7 days. The rats in group 3 received equivalent dose of blank cubosomal dispersion (negative control). The remaining group received no medication (normal control). Blood samples were collected from all rats after 7 days of treatment. Serum levels of liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were estimated by kinetic method using semiautomatic analyzer BTS-350 (BioSystems, Spain). At the end of the study, all the rats were sacrificed by cervical dislocation; livers were excised and transferred in 10% formalin saline solution for histopathological examination. Autopsy samples were taken from the liver of rats in the different groups and fixed in 10% formal saline for 24 h. Washing was done in tap water then serial dilutions of alcohols (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in a hot oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at thickness of 4 μm by sliding microtome. The obtained sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stain for routine examination through electric microscope\(^13\).

2.8. Statistical analysis
In order to compare the results, Student’s t-test (SPSS program; version 12.0) was used. Stability data were compared using paired t-test. Data reported as mean±standard deviations (SD). A statistically significant difference was considered at \(P < 0.05\).

3. Results and discussion
3.1. Preparation of cubosomes
Blank and 5-FU-loaded cubosomal nanoparticle dispersions were prepared through disrupting a cubic gel phase of GMO and water in the presence of poloxamer 407 as a stabilizer by mechanical stirring. The dispersions appeared as uniform opaque white mixtures with no visible signs of aggregate. The final concentration of lipid in the dispersion was 10% w/v with respect to the final dispersion weight. The ratio of GMO to poloxamer 407 in total lipid content was 9:1 w/w. The choice of this ratio was based upon observations of Jin et al.\(^32\) who found that cubosomes of this composition have reasonable physicochemical properties and improved the absorption of a poorly absorbed drug.

3.2. Characterization of cubosomes
The morphological examination of 5-FU cubosomal nanoparticles was performed using TEM. The photograph (Fig. 1) reveals that the drug-loaded cubosomal nanoparticles are nearly spherical with irregular polyangular shapes without aggregation. However, the particle diameters are smaller than those showed by particles size measurement determined by a dynamic light scattering particle size analyzer.

EE (%) of 5-FU in cubosomes was determined after separation of the free 5-FU from cubosomal nanoparticles loaded with the drug by ultrafiltration centrifugation. Drug adsorption to the ultrafiltration membrane was insignificant as reflected by nearly 100% recovery of the tested drug concentrations. The EE (%) was (31.21 ± 2.83)%, which revealed that most of the drug was not entrapped in the cubosomes. Similar EE (%) values of 5-FU were previously reported with other lipid based vesicular delivery systems of 5-FU such as niosomes\(^28\) and liposomes\(^33,34\). The low EE (%) of 5-FU may be attributed to the extensive mobile character of the small 5-FU molecule, which does not associate with the lipid bilayer\(^25\). Moreover, due to the hydrophilic nature of 5-FU molecules (logP<sub>octanol/water</sub> = –0.89)\(^20\) and its limited solubility (approximately 0.13%, w/v) in GMO, 5-FU was expected to be entrapped within the aqueous channels of cubosomal
nanoparticles. These conditions might favor the rapid leakage of the drug from the aqueous channels to the surrounding aqueous phase during the preparation and centrifugation processes. Previous studies reported that even lipophilic drugs were rapidly released from cubosomes after ultrafiltration.

The results of particle size and polydispersity index of the prepared cubosomes are presented in Table 1. Mean particle sizes of blank and 5-FU-loaded cubosomes were 91.28 ± 4.54 nm and 105.70 ± 5.47 nm, respectively. Both dispersions displayed a narrow monomodal particle size distribution (Fig. 2). The value obtained for particle size may be attributed to the use of a relatively high concentration of poloxamer 407 (10%, w/w relative to the dispersed phase) as a stabilizer. The polydispersity indices of blank and 5-FU-loaded cubosomal dispersions were 0.343 ± 0.056 and 0.429 ± 0.154, respectively. These relatively high values for both dispersions may be attributed to the coexistence of cubosomes with other type of vesicles in the cubosomal dispersion as previously reported. The presence of predominantly vesicular structures at high poloxamer 407 concentrations may be due to the formation of mixed monoolein/poloxamer bilayers which sterically stabilize the particles against their fusion into the cubic state. Although comparatively high poloxamer 407 concentrations are beneficial in terms of formation of smaller particles, they also promote formation of vesicular particles over formation of the desired particles of cubic structure.

Fig. 3 shows the DSC thermograms of 5-FU, GMO, poloxamer 407, blank cubosomes and 5-FU-loaded cubosomes. It is clear that the DSC thermogram of 5-FU exhibits a single sharp characteristic, endothermic melting peak at 281.6 °C which is in agreement with that reported previously. However, 5-FU melting peak completely disappeared in thermogram of 5-FU-loaded cubosomes. This indicates that the drug incorporated in the cubosomes existed in a non-crystalline state.

X-ray diffraction (Fig. 4) was carried out to confirm the physical state of 5-FU loaded into cubosomes in comparison to drug-free cubosomes, pure 5-FU, GMO and poloxamer 407. It is clear that the diffractogram of the pure 5-FU exhibited characteristic intensity reflections counts of 898, 1448 and 706 at diffraction angles of 9.51°, 29.79° and 33.47° (2θ), respectively, indicating its crystalline nature. However, these characteristic peaks disappeared in the X-ray diffraction pattern of 5-FU-loaded cubosomes. Moreover, the powder X-ray diffraction pattern for the drug loaded cubosomes was without any remarkable difference when compared to the powder X-ray pattern for blank cubosomes. This indicates that the drug was molecularly dispersed or in non-crystalline state and confirms previous results from the DSC analysis.

Results of 5-FU in vitro release from 5-FU-loaded cubosomes compared to 5-FU aqueous solution are illustrated in Fig. 5A. A rapid and complete release from 5-FU aqueous solution was obtained after 1 h. The release profile of 5-FU from cubosomes was biphasic, with an initial burst release of approximately (53.60 ± 3.55)% of drug during the first hour, followed by a relatively slow drug release of the remaining drug after 4.5 h. The higher initial burst release is mainly attributed to weakly bound or previously.

### Table 1

| Formulation                  | EE (%)          | Particle size (nm) | PDI            |
|------------------------------|-----------------|--------------------|----------------|
| Blank cubosomes              | –               | 91.28 ± 4.54       | 0.343 ± 0.056  |
| 5-FU-loaded cubosomes        | 31.21 ± 2.83    | 105.70 ± 5.47      | 0.429 ± 0.154  |

Data are expressed as mean ± SD, n = 3.
adsorbed drug to the relatively larger surface of nanoparticles\textsuperscript{44}. Moreover, in our case, the low affinity of 5-FU to the hydrophobic domain in the cubosomes made it easy to be released faster through diffusion from aqueous channels. The burst release of hydrophilic and hydrophobic drugs from cubosomes was previously reported\textsuperscript{38,45}. On the other hand, the relatively slow release of 5-FU observed from cubosomes may be attributed to the limited diffusion of drug molecules incorporated in the aqueous channels; in this case diffusion is governed by the tortuosity and the relatively narrow pore size of the aqueous channels\textsuperscript{46,47}. The potential of cubosomes to provide a slow release matrix for drugs of varying sizes and polarity has been reported\textsuperscript{48–52}. However, the present results demonstrate that the overall release of 5-FU from cubosomes is relatively rapid compared to the reported slow and sustained release of other drugs incorporated into cubosomes\textsuperscript{53,54}.

It should be noted that the latter drugs are considerably more lipid soluble than 5-FU, a water soluble compound. The presently-documented release of cubosomal 5-FU is also rapid as compared with the release of 5-FU from other drug delivery systems\textsuperscript{6–11}.

To emphasize the diffusion controlled release of 5-FU, the release data were plotted vs. the square root of time (Fig. 5B). A linear relationship was found for both free 5-FU aqueous solutions and 5-FU-loaded cubosomes with correlation coefficients of 0.985 and 0.992, respectively, indicating that the diffusion is the dominant mechanism of release\textsuperscript{55}. These results are consistent with other reports of drug release from cubosomal nanoparticles\textsuperscript{49,50,56}.  

3.3. Stability study

Studies of in vitro release revealed that the release of 5-FU from the cubosomal nanoparticles is quite rapid; this could limit the storage time of the cubosomal dispersion. Therefore, the cubic gel containing 5-FU was prepared and stored until disrupted with water using vortex to prepare the cubosomal dispersion just before its use. After 3 months of storage of the cubic gel containing 5-FU at refrigeration temperature (4–8 °C), the cubosomal dispersion was prepared to measure the particle size and EE (%). The mean particle size (± SD, n = 3) increased from 105.70 ± 5.47 nm to 112.34 ± 2.6 nm. The mean EE% (± SD, n = 3) decreased from (31.21 ± 2.83)% to (29.11 ± 0.62)%). The slight increase in the mean particle size and decrease in EE (%) were found to be statistically insignificant (P > 0.05, paired t-test), indicating that storage of cubic gel in tightly closed amber glass containers at refrigerator temperature (4–8 °C) did not adversely affect either the particle size or EE (%) of the prepared cubosomal dispersion. The ability of the GMO cubic phase gel to protect small, labile drugs (such as cefazolin and cefuroxime) from chemical instability reactions (such as hydrolysis and oxidation) was previously reported\textsuperscript{57}.

3.4. In vitro cytotoxicity of 5-FU-loaded cubosomes

Cytotoxicity of 5-FU cubosomal formulation was evaluated in human hepatoma HepG2 cell line and compared to the effects of blank cubosomes and free 5-FU aqueous solutions. The concentration-dependent cell viability curves are presented in Fig. 6. The half-maximal inhibitory concentrations (IC\textsubscript{50}) were 107.78 µg/mL and 112.70 µg/mL for 5-FU cubosomal dispersion and free 5-FU, respectively. The difference between the mean IC\textsubscript{50} values was found to be statistically insignificant (P > 0.05) when analyzed using student’s t-test. Accordingly, 5-FU cubosomal formulation has an equally efficient cytotoxic activity as compared...
3.5. Biodistribution of 5-FU in rat’s liver

The mean 5-FU concentration in rat liver tissues at various time intervals after subcutaneous injection of a single dose (10 mg/kg) of free 5-FU solution and cubosomal dispersion is shown in Fig. 7. In the case of 5-FU solution, after 1 h, the liver 5-FU concentration was 7.14 ± 1.52 μg/g and declined rapidly to 3.94 ± 0.46 and 1.70 ± 0.24 μg/g after the 2nd or 3rd hour, respectively. Such rapid decline of 5-FU level might have resulted from the highest accessibility of free 5-FU to its metabolizing enzymes. On the other hand, 5-FU-loaded cubosomes showed a gradual increase of 5-FU liver concentration from 4.70 ± 0.85 μg/g at the first hour to 6.04 ± 1.02 and 8.40 ± 1.66 μg/g after 2 and 3 h, respectively. The 5-FU concentration in liver at 3 h after subcutaneous administration of cubosomal formulation was nearly 5-fold that observed in case of 5-FU solution. The higher 5-FU liver concentration associated with the cubosomal formulation might be due to the higher systemic absorption of the 5-FU-loaded cubosomal nanoparticles from subcutaneous tissues. The enhanced systemic absorption of cubic nanoparticles-associated drugs might be attributed to the higher permeability of the epithelial membrane to cubosomes as a result of the structural similarity of the lipid bilayer of cubosomes to the microstructure of the cell membrane58,59. Moreover, the systemically circulated 5-FU-loaded cubosomal nanoparticles are susceptible to preferential phagocytic uptake of the reticuloendothelial system in the liver tissues. These results suggest that cubosomal formulation of 5-FU may exhibit therapeutic activity in the liver for a prolonged period compared to the aqueous solution.

The size of a nanoparticle is very important for drug delivery, as the spaces between the cells in various tissues are different. For example, it is now known that the aperture of the vascular endothelium within most normal tissues is 2 nm, and the aperture of the postcapillary venules is 6 nm. In contrast, the aperture of non-continuous tumor blood vessels ranges from 100 nm to 780 nm60,61.

Therefore, in treatment of hepatocellular carcinoma, the size of 5-FU-loaded cubosomal nanoparticles (105.70 ± 5.47 nm) could allow the nanoparticles to enter the space within tumor cells but restrict drug penetration into normal tissues. This is expected to enhance the efficacy and minimize the systemic side-effects of 5-FU in treatment of liver cancer.

3.6. Liver function and histopathological alteration

3.6.1. Liver function

5-FU was found to produce liver toxicity associated with a number of abnormalities62. Serum levels of hepatic enzymes (AST and ALT) were determined to evaluate the liver function after treatment of rats with 5-FU cubosomal dispersion, blank cubosomes and free 5-FU solution. An abnormal rise in either AST or ALT levels indicates liver dysfunction or damage. From Table 2, levels of AST and ALT were significantly (P < 0.001) increased in rats treated with either 5-FU-loaded cubosomes or free 5-FU solution when compared with those in the normal and negative control groups, indicating the toxicity of 5-FU on the liver in both groups. However, rats treated with 5-FU-loaded cubosomes developed higher hepatocellular damage as evident from the significantly (P < 0.001) higher levels of AST and ALT when compared with rats treated with free 5-FU. The significant elevation in AST and ALT levels would be attributable to the higher 5-FU concentration in hepatic tissues of rats treated with
cubosomal formulation. Therefore, histopathological examination of livers from treated and control groups was performed for further evaluation.

3.6.2. Histopathological alterations
The histopathology of the liver tissues of control, 5-FU cubosomal formulation and free 5-FU treated rats is shown in Fig. 8. The histology studies of control liver (Fig. 8A) showed a normal histological structure of the central vein (CV), portal area (A) and surrounding hepatocytes (H). In contrast, rats treated with 5-FU-loaded cubosomes (Fig. 8B and C) showed severe dilation and congestion in central vein (CV) and portal vein (PV) as well as hepatic sinusoids (S). In addition, fatty changes in some of the hepatocytes (arrow) and infiltration of inflammatory cells between the hepatocytes in the portal area were also noted. As compared with the effects of 5-FU-loaded cubosomes, the free 5-FU treated group showed only mild congestion in the central and portal veins (Fig. 8D) associated with fatty changes and ballooning degeneration in the hepatocytes (arrow, Fig. 8E) at the periphery of the hepatic parenchyma. This pattern suggests lower hepatotoxicity from the free 5-FU group as compared with the 5-FU-loaded cubosome group. The severity scores of histopathological alterations in the livers of different groups (Table 3) were moderate to severe in rats treated with 5-FU cubosomal formulation and were mild to moderate in rats treated with free 5-FU compared to control rats. These results indicate that the cubosomal formulation increases the hepatotoxicity of 5-FU that could be attributed to the higher 5-FU concentration in the liver tissues. These results were in contrary with Cheng et al.10 who demonstrated that the damage of liver function caused by 5-FU can be reduced when 5-FU was formulated as galactosylated chitosan/5-FU nanoparticles. However, further studies are required to evaluate hepatotoxicity and in vivo antitumor activity of lower doses of 5-FU cubosomal formulation in treatment of hepatocellular carcinoma.

4. Conclusions
5-FU, a hydrophilic anticancer drug, was successfully incorporated into cubosomal nanoparticles. Cubosomes loaded with 5-FU exhibited a nanometer-size particles with narrow particle size distribution. In vivo, biodistribution studies of 5-FU in rat liver indicated that the cubosomal formulation significantly increased...
5-FU liver concentration (nearly 5-fold) as compared to that of a 5-FU solution. On the other hand, histology studies indicated that the increased 5-FU concentration in the liver tissues resulted in a higher hepatocellular damage. Based on the previous results, the use of cubosomes as a drug delivery system is expected to improve the efficacy of low doses of 5-FU. Further studies are required to evaluate hepatotoxicity and in vivo antitumor activity of lower doses of 5-FU in cubosomal formulations for the treatment of hepatocellular carcinoma.

Table 3  Severity scores of the histopathological alterations in the livers of different groups.

| Group                  | Congestion | Fatty change | Inflammatory reactions |
|------------------------|------------|--------------|------------------------|
| Control                | –          | –            | –                      |
| 5-FU cubosomal dispersion | +++       | ++           | +++                    |
| 5-FU solution          | +          | +            | –                      |

+++ Sever, ++ Moderate, + Mild, – Nil. n=3.

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