ORIGINAL ARTICLE

Double-loaded liposomes encapsulating Quercetin and Quercetin beta-cyclodextrin complexes: Preparation, characterization and evaluation

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Beta-cyclodextrin (CD) inclusion complexes of Quercetin were formed and characterized by Differential scanning calorimetry (DSC) and Fourier transform infra-red spectroscopy (FTIR) spectroscopy. Plain Quercetin liposomes using phosphatidylcholine and cholesterol were prepared and optimized. Factors such as ratio of lipids employed, drug:lipid ratio, etc. were fine tuned and optimized to achieve maximum entrapment of the Quercetin into the bilayer. Entrapment was further enhanced by double loading the liposomes. These were prepared by incorporating Quercetin as a plain drug as well as the inclusion complexes within the lipid bilayer and the aqueous compartment, respectively, of the liposomes using the thin film hydration technique. The highest entrapment was achieved with a lipid ratio of 9:1, and the amount of plain drug entering the bilayer was $1/10^{th}$ the amount of lipid employed. Double loading increased this value to one part of drug per five parts of lipid when Quercetin–beta-CD (1:1 mol/mol) was entrapped. The release of Quercetin from liposomes was highest when the drug was entrapped in the form of a complex with beta cylodextrin. The high entrapment ability of Quercetin in the form of plain drug as well as beta cylodextrin–Quercetin complexes in comparison with plain drug is an indubitable advantage of this approach.

Key words: *Cyclodextrin, drug entrapment, lipid peroxidation, liposome, Quercetin–beta-cyclodextrin complex*

INTRODUCTION

Liposomes are spherical vesicles composed of lipidic ampiphiles, usually phospholipids, which organize themselves in water to form an aqueous core surrounded by lipid bilayers. This unique structure allows the liposomes to encapsulate both hydrophilic and lipophilic materials.[1] In doing so, this carrier system protects the entrapped molecules from degradation and thinning down in the systemic circulation.[2] Because of their properties, liposomes when formed, their physiochemical properties like size, lamellarity, membrane rigidity, etc. are able to influence and enhance the performance of products by increasing ingredient solubility, improving ingredient bioavailability, enhance intracellular uptake, alter pharmacokinetics and biodistribution and *in vitro* and *in vivo* stability.[3] Liposomes are also known to prevent local irritation, increase drug potency and reduce toxicity.[4,5] However, the amount of drug that can be encapsulated within the liposomes impedes its employment as a carrier system. Both the properties of the liposome as well as that of the entrapped drug can manipulate the encapsulation efficiency.[6] Drugs may be incompatible with vesicle formation, and the accommodation of water-insoluble drugs in the lipid bilayers of the liposomes can be detrimental to the bilayer formation and stability and require the use of suitable organic solvents. Increasing the lipid load in order to incorporate sufficient drug for adequate therapeutic efficacy may not be acceptable, particularly with chronic use.[7] Simultaneous entrapment of the drug into the lipid bilayers as well as into the aqueous

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phase of the liposome by virtue of the water-soluble cyclodextrin (CD) inclusion complexes is a prospective approach for surmounting such shortcomings and combining the relative advantages of the two types of carriers into a single system by formulating drug-in cyclodextrin-in liposome (CLDDS) systems.

The concept of CLDDS was reported in 1994.[8,9] CDs are cyclic oligosaccharides of α-D-glucopyranose formed by the action of certain enzymes on starch. Complexation of drug compounds with CDs improves drug solubility, protects against the effects of light, heat and oxidation and masks the unwanted physiological effects associated with the drug molecule. CDs also have stabilizing effects and they have no effect on reactivity or on the degradation of the entrapped drug molecule. The CD molecule also partially shields the guest molecule from attack by various reactive molecules.[10]

The possibility of using such a combined strategy aimed to concurrently exploit the CD-solubilizing power toward the drug and the tailored release mechanisms offered by liposomes.

CLDDS could thus control the duration of drug action and the *in vivo* fate of the drug in a better manner.[11]

With regards other organ systems, the fact that the vast majority of liposomal systems are taken up by the liver is a serious problem in designing therapeutics. But, in case of liver diseases, the natural targeting of liposomes is of great potential value. Liposomes can be readily employed to decrease toxicity of therapeutic agents to other organs, while at the same time enhancing the drug concentration of these agents, which show effects on the liver.

Cell damage resulting from the actions of free radical species (FRS) and reactive oxygen species (ROS) has been indicated in the pathogenesis of liver diseases like jaundice and hepatitis.[12] These reactive species or oxidants are radicals that are atoms or molecules with at least one unpaired electron. They are highly reactive, allowing protein side-chains, lipids and DNA to be easily modified without the aid or presence of catalytic enzymes. This in turn leads to excessive molecular damage and tissue injury. Oxidative stress states occur when cellular antioxidant defenses are insufficient to keep the levels of ROS below a toxic threshold. This may be due to excessive production of ROS or the failure of antioxidant defenses, or both.[13]

Hence, we decided to formulate CLDDS for Quercetin, a well-established antioxidant. Quercetin, a pentahydroxy flavone, is by and large distributed widely in the plant kingdom. However, the inherent bioavailability of the molecule is very low.[14] This molecule has an aberrant solubility pattern and attempts to improve its bioavailability are plenty in the literature. Mulholland *et al*.[15] have synthesized a water-soluble derivative of Quercetin while Yan Gao *et al*.[16] have formulated it into microemulsion. A nanoparticulate delivery system for Quercetin has been designed; Dimethyl Sulfoxide (DMSO) has been used as a solubilizing agent for Quercetin[17] and polymer-based solubility enhancement of Quercetin[18] have been published. However, the net improvement in solubility with the proposed soluble derivative was a mere 20%, Quercetin microemulsion incorporated about 50% of the surfactants and the safety of the materials used for nanoparticle preparation as well as toxicity profile of DMSO is questionable. Liposomes of Quercetin have been formulated but for topical purposes. Hence, a stable carrier for delivering Quercetin to the hepatocytes is desired, warranting its delivery as CLDDS.

Toward this purpose, we report the simultaneous entrapment of Quercetin into the bilayer as well as the aqueous compartment of liposomes. This treatment effectively increases the drug:lipid ratios to levels higher than those obtained by entrapping only the drug in the liposomal bilayer. Because of the lipophilic nature of Quercetin, it gets embedded in the liposomal bilayer and the water-soluble CD complex enters the aqueous interior. The classic method of Bangham[19] was used to formulate the liposomes. All liposomal formulations were characterized for encapsulation efficiency, particle size, drug loading and morphology. *In vitro* release profiles were determined and an *in vivo* lipid peroxidation assay was performed to ascertain the effectiveness of the formulation developed.

MATERIALS AND METHODS

Materials

Quercetin was purchased from Fine Chemicals (Mumbai, India). Phosphatidylcholine was a gift sample from Lipoid (Ludwigshafen, Germany). Cholesterol was obtained from Sigma (St. Louis, MO, USA). β-CD was obtained from Gangwal Chemicals (Mumbai, India). HPLC-grade solvents were purchased from Merck (Mumbai, India). All other materials and solvents used were of analytical grade.

HPLC method development

Initial trial experiments were conducted with a view to select a suitable solvent system for the accurate estimation of Quercetin. A mobile phase system comprising of methanol–water (70:30% v/v) was found to be optimum. The solvents were mixed, filtered through a membrane filter of 0.45 micron pore and degassed before use. The chromatography system comprised of a Jasco PU-980 pump equipped with a Jasco UV-975 detector and a rheodyne injector with a 20-μL loop. Data integration was done using a Borwin software package V1.21. Samples were injected into a Hi-Q-Sil C-18 column (4.6 mm × 250 mm, 5 μ particle size). The mobile phase flow rate was 1 mL/min. Quercetin was analyzed at a wavelength of 256 nm.

Formation of Quercetin– β-cyclodextrin inclusion complexes

Phase solubility studies

Phase solubility measurements were carried out according to the method of Higuchi and Connors.[20] Excess amount of Quercetin (5 mg) was added to 5 mL of deionized water containing increasing amounts of β-CD (ranging from 0 to 0.020 M). The resulting mixture was equilibrated by placing the flasks on the rotary shaker at room temperature for 48 h. To minimize photochemical degradation, the flasks were covered with aluminum foil. Then, suspensions were filtered through a 0.45 µm cellulose acetate membrane filter to remove undissolved solid. An aliquot from each vial was adequately diluted and spectrophotometrically analyzed at 256 nm. Shaking was continued until three consecutive experiments yielded similar results.

The apparent stability constant (*K*s) of the complexes were calculated from the phase-solubility diagrams according to the following equation:

$$K_S = \frac{\text{Slope}}{S_0(1-\text{slope})} \quad (1)$$

Where S_0 is the solubility of Quercetin at 30°C in the absence of CD and slope means the corresponding slope of the phase-solubility diagrams, i.e. the slope of the drug molar concentration versus CDs molar concentration graph.

Method employed

Solid state Quercetin complexes with β-CD in 1:1 molar ratios were prepared according to the method described by Kurozumi *et al*.,[21] with some modifications. Quercetin (0.6 g) and β-CD (2.27 g) were accurately weighed and dissolved in about 150 mL of distilled water. To this aqueous solution, further, 25% ammonia (two to three drops) was added to dissolve the Quercetin. The whole solution was stirred on a magnetic stirrer for 4 h till a clear solution resulted. The solution was frozen overnight and then lyophilized. The free flowing solid was then passed through sieve #60 and stored in a dessicator.

Characterization of Quercetin–β-CD complexes

Differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR) were used to confirm the formation of Quercetin–beta -CD complexes. The samples of Quercetin, β-CD and the Quercetin–β-CD freeze-dried complexes were triturated with KBr and pelletized into discs. FTIR spectra were then recorded using a Jasco FTIR instrument.

DSC of Quercetin, β-CD and the freeze-dried complexes was carried out using a Mettler Toledo, DSC 821e. All the materials used were in the solid state. Typical conditions were: temperature range - 0°C to 350°C, scanning rate 10°C/min. Baseline optimization was performed before each run.

Preparation of Quercetin-loaded liposomes

Multilamellar vesicles consisting of a mixture of lipids (phosphatidylcholine and cholesterol in different ratios) were prepared according to the thin-layer evaporation technique using 2:1 v/v of chloroform:methanol.[19,22,23] In brief, methanolic solution of Quercetin was added to the lipid solution in chloroform. The solvents were removed under reduced pressure in a rotary evaporator (Superfit, Mumbai, India) thus obtaining a thin film of dried lipid on the flask wall. Evaporation was continued for 1 h after the dry residue appeared to completely remove all the traces of the organic solvent. The film was then hydrated by adding buffer (pH 5.7) under vigorous stirring in order to affect vesicle formation. The milky suspension containing multilamellar vesicles was further size reduced in a probe sonicator (Oscar, Mumbai, India).

Preparation of liposomes loaded with plain Quercetin and Quercetin–β-CD complexes (double-loaded liposomes)

Essentially, the same method of Bangham,[19] as stated above, was used to prepare double-loaded liposomes. However, while hydrating the lipid film with buffer, the Quercetin–β-CD complexes were dissolved in the buffer and then incorporated into the aqueous phase of the liposomes.[24] Thus, Quercetin was "double" loaded into the lipid bilayers as well as the aqueous compartment of the liposomes. This double loading was confirmed using DSC in accordance with the protocol stated earlier.

Determination of drug entrapment efficiency

Drug entrapment efficiency was determined by the centrifugation technique, which works in principle by determining the amount of nonentrapped drug from the liposomes.[22,25] The liposomal suspension was centrifuged at 15,000 rpm for 60 min (Eltek RC 4100 D, Elektrocrafts, Mumbai, India) to obtain the liposomal precipitate. The supernatant was analyzed by HPLC for determination of unentrapped Quercetin.

The precipitate was then collected and dispersed in 0.1% w/v sodium lauryl sulfate solution and surged by vortexing (REMI, Mumbai, India) for 3 min in order to dissolve the free Quercetin adsorbed onto the surface of liposomes. The resulting dispersions were centrifuged for 30 min at 15,000 rpm (Eltek RC 4100 D). The drug content in both supernatants after centrifugation was measured by the developed HPLC method using methanol:water (70:30% v/v) in triplicate.

The drug entrapment efficiency (EE%) of Quercetin liposomes was calculated as per the following equation:

$$EE\% = \frac{Wa - (Ws_1 + Ws_2)}{Wa} \times 100\% \quad (2)$$

Where, W_a = weight of drug added into the system,

Ws_1 =analyzed weight of drug in the first supernatant, Ws_2 = analyzed weight of drug in the second supernatant.

Determination of drug loading

The drug loading (DL%) was calculated according to the following equation:

$$DL\% = \frac{\text{Amount of drug entrapped}}{\text{Amount of drug added} + \text{amount of lipid added}} \times 100 \quad (3)$$

Morphology of liposomes (transmission electron microscopy

The ultrastructure of the liposomes was determined by using TEM (Philips CM 200, Andover, Massachusetts, USA). Samples were prepared by suitably diluting the formulation. Contrast was improved using 2% tungstophosphoric acid.[26] Samples were placed on copper grids coated with carbon film, and were left in contact for 15 min. The grids were then blotted on a filter paper and dried further by placing in a petriplate at room temperature, which was then analyzed with TEM and scanned for observation of vesicles.

In vitro drug release from Quercetin liposomes

Release studies were carried out according to the dialysis method.[27] The release studies were carried out in a 250-mL beaker containing 200 mL of the medium. The medium was stirred using a magnetic needle. Dialysis membrane (Hi-Media, India) was used as a barrier to isolate the donor and the receptor phase. Liposomal dispersion was placed in a dialysis bag (Hi-Media) of cellulose acetate, which was immersed in the medium and magnetically stirred.

The stirring was carried out at 100 rpm at 37°C ± 0.5°C. The simulation of GI transit condition was achieved by altering the pH of the dissolution medium at different time intervals. For the initial 2 h, the pH of the dissolution medium was adjusted to 1.2 using 0.1 N HCl. Subsequently, the pH of the dissolution medium was adjusted to 7.4 with 0.1 N NaOH and buffer salts, and maintained up to 20 h. Samples taken from the receiver solution at predetermined times were replaced with equal volumes of fresh buffer and spectrometrically assayed for drug content assayed for drug content by High Performance Liquid Chromatography (HPLC) (Jasco, Mumbai, India).

Ex-vivo lipid peroxidation studies

The method proposed by Ohkawa *et al*.[28] was used for estimation of inhibition of lipid peroxidation. Briefly, 10% liver homogenate (obtained freshly and homogenized with 0.15 M KCl) was mixed with 150 mM KCl and TRIS buffer. The test samples of plain Quercetin and Quercetin double-loaded liposomes were then added in various concentrations. *In vitro* lipid peroxidation was initiated by the addition of $FeSO_4$ (10 μM) and ascorbic acid (100 μM). After incubation for 1 h at 37°C, the reaction was terminated by addition of thio barbituric acid (TBA) reagent (2 mL) and boiled at 95°C for 15 min for the development of a colored complex. After cooling, the tubes were centrifuged at 4000 rpm for 10 min. The absorbance of supernatant was determined colorimetrically at 532 nm. Percentage inhibition of TBA-reacting substances (TBARS) formed were calculated with respect to the control in which no test sample was added. The inhibition of lipid peroxidation was determined by calculating the % decrease in the formation of TBARS, and the IC_{50} was calculated.

Calculation

The percent inhibition of lipid peroxidation of test/standard drug was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (4)$$

Where, A_0 is the absorbance of the control (blank) and A_1 is the absorbance in the presence of the test samples.

RESULTS

Formulation of Quercetin– β-cyclodextrin complexes

Phase solubility diagrams obtained with β-cyclodextrin (BCD) showed a linear relationship between the amounts of Quercetin solubilized and the concentration of CD in the solution (AL type diagram). According to Higuchi and Connors' theory, this may be attributed to the formation of soluble 1:1 Quercetin–beta-CD inclusion complexes. The stability constant was found to be 502.51/M. These results were in accordance to a similar study conducted by Tayade and Kale.[29]

The formation of Quercetin–β-CD complex was ascertained using DSC and FTIR. The DSC spectrum of the complex [Figure 1] reveals the characteristic absence of the melting peak of Quercetin at 310°. The complete disappearance of the drug endothermal peak was instead observed for systems obtained by freeze-drying. This phenomenon can be assumed as proof of interactions between the components of the respective binary systems.[30] This can be considered as indicative of drug amorphization and/or inclusion complex formation.

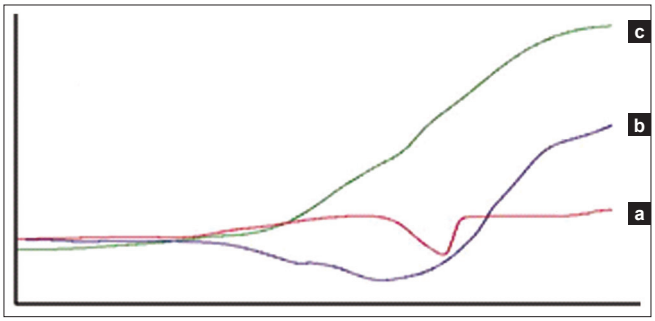

Figure 1: DSC spectrum showing the formation of Quercetin- and α-cyclodextrin complex. DSC spectrum of (a) Quercetin (b) and α-cyclodextrin and (c) Quercetin- and α-cyclodextrin complex

The formation of these complexes was further corroborated using FTIR. Infrared spectra (not shown) of Quercetin crystals revealed a characteristic carbonyl absorption band at 1661.75/cm, assigned to aromatic ketonic carbonyl stretching. In the case of freeze-dried products, in particular, the characteristic aromatic carbonyl stretching band of drug appeared shifted to 1629.29/cm for Quercetin-beta-CD complex along with reduced intensity of the same band. There was no change in the spectra of physical mixtures. Changes in the characteristic bands of pure drug confirm the existence of the complex as a new compound with different spectroscopic bands.[31]

Formulation of Quercetin-loaded liposomes

Initially, the liposomal formulations (with various PC:CH and drug:lipid ratios) were studied in light of their entrapment efficiency, % drug loaded and particle size, which revealed the profound interaction of the drug with the lipid bilayers enabling the screening of the batches for double loading. The entrapment efficiencies were higher for the batches formulated with a lipid ratio of 9:1. Batch with drug lipid ratio of 1:10 further yielded the maximum entrapment of 82.64 ± 1.31%. The mean size of the liposomes ranged from 131.7 ± 6.37 to 617.3 ± 8.85 nm. Formulations having a particle size in the range of 200–400 nm were further selected for double loading. This would ensure that the particles would be sufficiently small as well as capable for double loading. Increasing the drug content above 1/10th the lipid content led to sedimentation of the formulation probably because of the low entrapment and hence the settling of these particles, rendering the formulation unstable.

Formulation of Quercetin double-loaded liposomes

In the DSC thermogram [Figure 2] of double-loaded Quercetin liposomal dispersion, the cholesterol endotherm exhibited a slight shift from ~147°C to ~139°C. Absence of the melting endotherm of Quercetin suggested significant interaction of Quercetin with the bilayer structure. The above observation is concordant with earlier findings that interaction of the encapsulated active with the lipid components of liposomes may alter the physicochemical properties of liposomes, which in turn would influence the transfer of the active from the liposomes.[32]

The characteristics of the double-loaded liposomes are as shown in Table 1. The values for encapsulation efficiencies of the double-loaded liposomes were in the range of 85.73 ± 0.60 to 90.68 ± 0.51%. These values indicated that a maximum entrapment was achieved when the drug to lipid ratio was 1:5. Drug loading was determined in order to ascertain the enhanced entrapment efficiency of the double-loaded batches over the plain drug-loaded batches. As expected, the values of %DL (expressed in terms of amount of drug entrapped to the amount of lipid added) was 90.23 ± 0.51 for the double-loaded liposomes in comparison with the plain drug-loaded batches, which amounted to a maximum of 81.82 ± 1.30 as shown in Table 1. Results signify

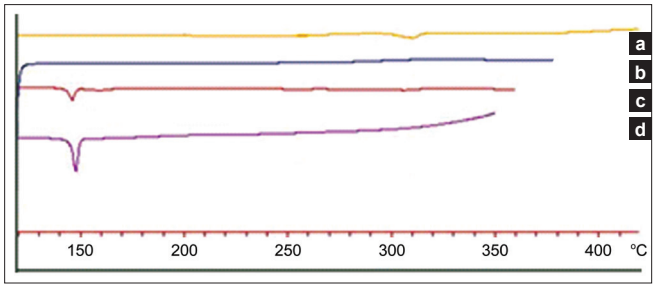

Figure 2: DSC spectrum showing the formation of Quercetin double loaded liposomes. DSC spectrum of (a) Quercetin, (b) Double loaded liposomes, (c) physical mixture of cholesterol, phospholipid and cyclodextrin and (d) cholesterol.

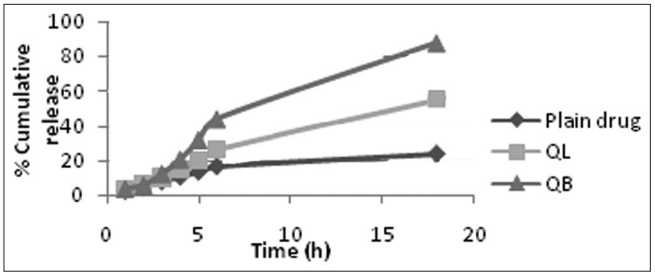

Figure 3: *In vitro* release profile of Plain Quercetin, Quercetin liposomes and Double loaded liposomes

Table 1: Composition, encapsulation efficiency, % drug loading and mean particle size of Quercetin-loaded liposomes and the corresponding Quercetin double-loaded liposomes (mean±SD, *n* = 3)

Batch code	Lipid ratio	Drug:lipid ratio	Encapsulation efficiency (%)	Drug Loading (%)	Mean particle size (nm)
Quercetin-loaded liposomes					
1	9:0	1:15	63.14±5.20	62.30±5.14	131.7±6.37
2	9:1	1:15	64.65±3.08	63.69±3.03	134.4±6.56
3	9:0	1:10	77.9±3.08	77.22±3.05	261.4±5.26
4	9:1	1:10	82.64±1.31	81.82±1.30	281.8±7.83
Quercetin double-loaded liposomes					
1	9:0	1:6	85.73±0.60	85.27±0.59	289±39.16
2	9:1	1:6	87.29±0.51	86.77±0.50	269±31.21
3	9:0	1:5	86.45±0.39	86.06±0.38	295±47.41
4	9:1	1:5	90.68±0.51	90.23±0.51	271±32.34

that the excess amount of Quercetin incorporated into the liposomes in the form of water-soluble CD complexes were successfully encapsulated and the loading capacity of the carrier was enhanced.

Release studies

The *in vitro* release graphs of Quercetin from the liposomes through the pH of the GIT are as shown in Figure 3. All the batches exhibited biphasic release profile and an initial burst release during the first 5 h followed by a continuous and slower sustained release. As expected, encapsulation of Quercetin into liposomes led to a controlled release rate due to the well-known reservoir effect of liposomes, with the release profile being identical in all cases.

Ex-vivo lipid peroxidation studies

Quercetin-loaded liposomes elicited concentration-dependent inhibition of $FeSO_4$-induced lipid peroxidation in rat liver homogenate. The IC_{50} value of the double-loaded liposomes were found to be 62.335 ± 13 μg/mL and that of plain drug-loaded liposome was 126.94 ± 52 μg/mL, whereas only Quercetin showed an IC_{50} value of 3198.86 ± 43 μg/mL. Results as shown in Figure 4 indicate that the double-loaded liposomes showed better protection against free radical damage induced by lipid peroxidation as compared with plain drug as well as plain drug-loaded liposomes.

DISCUSSION

Quercetin is a polyphenolic flavanol distributed ubiquitously in the plant kingdom. Most of the pharmacological effects of Quercetin are attributed to its ability to act as an antioxidant. It acts by inhibiting the initiation of the oxidation chain reaction and by preventing its propagation.[33] However, clinical studies investigating these effects have not translated well to show beneficial effects mainly due to the anomalous solubility profile of the molecule. Firstly, the molecule in the plants is present in the form of its glycosides while the actual activity is shown by the aglycone molecule. In the glycosidic form, the molecule is highly hydrophilic, which causes lower absorption of the molecule. Further, although the polyphenolic structure of the aglycone is highly beneficial to scavenge free radicals, it highly compromises the aqueous solubility of the molecule. Only about 25% of the ingested dose of Quercetin is absorbed from the small intestine.[14]

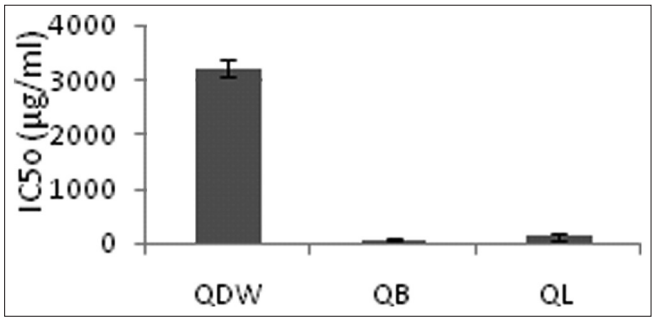

Figure 4: *Ex-vivo* Lipid peroxidation studies - IC50 values of Plain Quercetin, Quercetin Liposomes and Quercetin loaded liposomes

The literature search on Quercetin reveals that the absorption of the molecule is improved in the presence of lipids. The elimination of Quercetin is delayed when the molecule is administered with a fatty diet.[34] Also, Quercetin exhibits a high affinity for liposomes that results from its planar configuration, which can easily intercalate into the organized structure of the phospholipids within the vesicle membranes.[35] Phosphatidylcholine is the most commonly used phospholipid that makes up the lipid bilayers structure of liposomes plus it has proven hepatoprotective effects.[36] Taira *et al*.[37] have shown that phosphatidylcholine is stable at pH 1.2. Hence, we decided to formulate liposomes of Quercetin using phosphatidylcholine. Uncomplexed α- and β-CDs have been studied for their direct antioxidant activity, and a 2.3 μM CD solution provides better protection against lipid peroxidation, which is more evident for β-CD.[38,39] β-CD is a simple and suitable complexing agent for antioxidant guest molecules with innate antioxidant effects by virtue of its chelating properties and can thus enhance the antioxidant effects of the β-CD-entrapped drug molecule. To warrant a synergistic antioxidant effect with Quercetin, phosphatidylcholine and β-CD were preferred as components for the CLDDS.

Quercetin in the aglycone form is contemplated to penetrate the lipid bilayer by intercalating between the flexible acyl chains of the phospholipids. Electron paramagnetic resonance spectroscopy has shown that the planar Quercetin aglycone inserts more deeply into the membrane bilayer than the corresponding glycoside. In other words, the deepness of flavonoid insertion in the bilayer is modulated by their hydrophilicity.[40]

Movileanu *et al*.[41] and Priprem *et al*.[42] have reported better entrapment of Quercetin in the lipid bilayers provided that it is incorporated into the lipid bilayers during film formation, rather than its incorporation into the aqueous compartment of the liposomes. This can be attributed to the apparent lipophilicity of the molecule (log *K*oct/water = 1.81 ± 0.17) and in light of the results from established studies, Quercetin was incorporated in the lipid phase of the formulation.

In the present study, the effect of cholesterol was first studied on the bilayer integrity. The formation of the lipid bilayer and its fluidity is accounted by the amount of cholesterol inserted between the phosphatidylcholine molecules. Cholesterol confers sufficient flexibility to the bilayer and also contributes to proper release of the entrapped moiety, especially of drugs like Quercetin that interact with the lipid bilayer.[43] Highest entrapment was obtained with the batches having phosphatidyl choline:cholesterol ratio 9:1. This could be because the addition of cholesterol in this ratio provides optimum rigidity to the bilayer. Increase in the amount of cholesterol causes marked decrease in entrapment, probably

due to the fact that cholesterol might be replacing Quercetin in the bilayer.

Addition of lipophilic drugs within the liposomal barrier can, after a threshold level, disrupt the bilayer integrity and form mixed micelles, or can cause permeability changes in the bilayer membrane.[44] Hence, the maximum amount of drug that can be entrapped to form stable bilayer vesicles was evaluated, which was found to be 1/10th the amount of lipid employed. Further, increasing the drug amount led to irreversible sedimentation of the system. This can be postulated because of leakage of excessive drug caused due to alteration in the permeability of the bilayer. Based on the above observations, batches with drug to lipid ratio of 1:15 and 1:10, with phosphatidylcholine:cholesterol ratio of 9:0 and 9:1, were selected for double loading with CD complexes.

TEM analysis reveals that the liposomes are spherical in shape, but differ in size. The size of the double-loaded liposomes roughly ranges in the order of 269 to 295 nm. Furthermore, the images depict that all particles are similar in size in each system and do not aggregate. This also indicates that the system is monodispersed. TEM images also reveal formation of multilamellar vesicles with the bilayer membrane intact. This shows that the inclusion of CD–drug complex within the liposome did not affect the bilayer and lamellar integrity. Further, the particle size in case of plain drug-loaded liposomes as well as double-loaded liposomes are almost similar. There was in fact a slight increase in particle size in the double-loaded batches with drug:lipid ratio of 1:15 as compared with the plain drug-loaded liposomes. The batches with drug:lipid ratio of 1:10 however had similar values for particle size. While some authors have reported an increase in the size of CD-loaded liposomes,[45] our findings are in accordance with Fatouros *et al*.[46] and Skalko *et al*.,[47] who report only slight changes in liposomal size. The reason attributed in most of the cases was difference in the method of analyzing particle size and the formulation procedure as such. Hence, the influence of these parameters on particle size needs further tuning and research is warranted.

Entrapment efficiencies of up to 90.68 ± 0.51% were achieved for the double-loaded liposomes. Entrapment values were again linked to the ratio of lipids used in the system. Maximum drug was encapsulated by the double-loaded liposomal batch corresponding to the plain drug-loaded batch, which showed maximum entrapment with a lipid ratio of 9:1. The stated observation was corroborated with a corresponding enhancement in the values of percentage drug loading. Because of double loading of liposomes with Quercetin and Quercetin–beta-CD complexes, the ratio of Quercetin:lipid increased and hence the consequent increase in %DL.

Release data indicate that liposomes containing Quercetin–beta -CD complex release more drug than do liposomes encapsulating plain drug, drug release being related to entrapment efficiency and drug loading. It may be possible that during complex entrapment in liposomes, phospholipids or cholesterol enters the hydrophobic cavity of the empty CD molecules present in the liposomes' aqueous phase, or perhaps replaces the drug when the cavity contains drug molecules. Perhaps, Quercetin may be released both in free form as well as in the complexed form from the aqueous compartment. This release would then be attributed to the dissociation of Quercetin from the complex, followed by its partitioning into the lipid bilayers at rates dependent on its partition coefficient.[9,12]

The release of Quercetin from the double-loaded liposomes was also compared using free drug as a reference. The release from plain drug was a minimal 23.65% as compared with 55.09% for plain drug-loaded liposomes and 87.91% for double-loaded Quercetin liposomes. A closer look at the release graphs indicate that there is an initial rapid release for the first 5 h in all the three cases, followed by a sustained effect in the order plain drug > plain drug-loaded liposome > double-loaded liposome. While the lower solubility of the plain drug can account for its lower cumulative release, the release pattern of both the plain drug-loaded liposome and the CD-loaded liposome are almost similar, suggesting that double loading does not interfere with the release pattern of Quercetin. The improved release of the double-loaded liposome in lieu of the plain drug-loaded liposome can then be conveniently attributed to the higher drug:lipid ratio in the former.

Influence of CDs on the bilayer stability is of major concern. Bouldemarat *et al*.[48] have reported that the CDs, especially the methylated forms, are known to destabilize the lipid membrane of the liposomes. This was reasoned as cholesterol might be replacing the drug in the β-CD cavity. However, for β-CD to effectively function as a destabilizing agent, it should be present in the free form. In our system, it is present as a 1:1 stable complex with Quercetin, and this complex has an appreciable stability constant of 502.51/M. Hence, the possibility of instability occurring in the system during storage is ruled out, if the system is adequately freeze-dried and stored appropriately under low temperatures.

This equilibrium is bound to be disturbed once Quercetin is released from the CD cavity and the cavity is emptied. This will then lead to extraction of Quercetin to a large extent as well as cholesterol. However, the extent of cholesterol extraction would be low as a lower concentration of cholesterol with respect to the phospholipids in the bilayer prevents complexation of the latter and the multilamellarity of the investigated liposomes might partially preclude empty β-CD from approaching the bilayer and extract the cholesterol embedded in the internal lamellae. As a result, immediate lysis or dissolution of the liposomes would not occur, effecting sustained release. Lipid bilayers losing cholesterol will thus largely remain intact but become more fluid and/or permeable for release of Quercetin.[49]

Imbalance in the production of ROS such as superoxide, hydrogen peroxide, hydroxyl, alkoxyl and peroxyl radicals and the resident antioxidant defenses of the body against ROS causes oxidative stress, which in turn damages tissue and amplifies it by releasing prooxidative forms of reactive iron that are able to drive lipid peroxidation. A wide variety of techniques have been used to show that lipid peroxidation increases in many disease states and in tissues poisoned by a variety of toxins. Behind many of these reports is the unspoken assumption that the disease or toxin causes increased lipid peroxidation, which is then responsible for the toxicity. Measurement of lipid peroxidation may therefore be an excellent marker of tissue damage.[50]

Antilipid peroxidative assay revealed that double-loaded liposomes were successful in combating ROS-mediated damage and the ensuing toxicity. IC_{50} value is indicative of the concentration of the drug that is required to elicit 50% of the proposed activity. The IC_{50} values of double-loaded liposomes indicated that the anti-lipid peroxidative activity was almost doubled in comparison with the plain drug-loaded liposomes, and was strikingly 50-times more than the activity elicited by Quercetin suspension in distilled water. This is in agreement with the previous findings as reported by Terao *et al*.[51] and Ioku *et al*.[52]

CONCLUSIONS

Water-soluble CD complex solutions of Quercetin could be entrapped into the aqueous phase of stable multilamellar liposomes, in addition to incorporation of Quercetin into the liposomal bilayer by the thin film hydration method. DSC, IR and TEM measurements suggested that the Quercetin CD complexes were successfully loaded into the liposomes without affecting their morphology. Percent drug loading was almost doubled in the double-loaded liposomes when compared with the batches not containing the CD complexes. The liposomes containing CD inclusion complexes released more drug than did the liposomes encapsulating plain drug, the drug release being related to entrapment efficiency, which is in accordance with the previously stated results by Malaekeh-Nikouei and Davies.[53]

The present work was a basic attempt to combine the two delivery systems, namely liposomes and CDs. The initial selection of lipid and CD was done on the basis of the evidence in the literature citing the antioxidant effects of beta-CD and the hepatoprotective effects of phosphatidylcholine, to achieve synergistic effects with the antioxidant molecule Quercetin. Although *in vitro* release profiles of Quercetin double-loaded liposomes across the pH changes in the GIT have been established, the question of gastric stability of the liposomal formulations *in vivo* needs to be given due consideration. Betagiri and co-workers have attempted coating of the liposomal membrane with eudragits,[54] phthalates[55] and other polymers,[56] leading to improved stability of the system in the GIT. These findings could be usefully exploited to suitably modulate and control release rate. Depending on the need for a modulated drug release, different coatings could be applied on the surface of the already double-loaded liposomes with the final aim of improving its clinical effectiveness.

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