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Research Article

PREPARATION, OPTIMIZATION AND *IN-VIVO* HEPATOPROTECTIVE EVALUATION OF QUERCETIN LIPOSOMES

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ABSTRACT

The present study is aimed at the overall improvement in the bioavaialability and the resulting hepatoprotective potential of Quercetin. Liposomes of Quercetin using phosphatidylcholine and cholesterol have been prepared using the thin film hydration technique under vacuum conditions. Various factors affecting the entrapment of Quercetin into the lipid bilayers were evaluated using experimental techniques. Factors like drug:lipid ratio, pH, amount of Quercetin etc. were fine tuned to obtain optimized batches. The prepared liposomes could be used for controlled drug release of the antioxidant Quercetin. The drug-entrapped liposomes were characterized by transmission electron microscopy (TEM). Differential Scanning Calorimetry (DSC) studies were used to confirm the entrapment of the drug into liposomes. The drug loaded liposomes are spherical in shape and the optimized batch had average diameter of about 270 nm. Drug release study showed that the release of Queretin from liposomes improved and sustained effect with compared to plain drug. *Ex-vivo* lipid peroxidation assay to judge the antioxidant potential indicated that the liposomal preparation strikingly demonstrated almost 50 times more activity as compared to that elicited by Quercetin suspension in distilled water. The formulation also demonstrated superior hepatoprotective effects in rodents subjected to CCl4 induced hepatotoxicity.

Keywords: Quercetin Liposomes.

INTRODUCTION

Oxygen is an element that is indispensible for life. During the process of respiration, the oxygen that we breathe is reduced to water thereby generating cellular energy. However, this process also generates unavoidable products of mitochondrial respiration in the form of Reactive Oxygen Species (ROS). ROS are partially reduced reactive oxygen species that are capable of initiating free radical chain reactions that can cause damage in the form of peroxidation of membrane lipids, oxidative damage to nucleic acids and carbohydrates and the oxidation of sulfhydryl and other susceptible groups in proteins [1-4]. Oxygen derived free radicals appear to possess the propensity to initiate and promote carcinogenesis [5]. Free radicals can also be generated through environmental stimuli which eventually lead to oxidative stress. Free radical mediated injury has been postulated in the development and pathogenesis of a large number of diseases such as liver injury [6], diabetes [7], heart disease [8], cancer [5], ageing [2] etc. Antioxidant therapy is hence indicated under these circumstances.

Antioxidants are the molecules that help prevent, delay or repair the damage caused by oxidative stress. Quercetin is a polyphenolic flavanol distributed widely in the plant kingdom. It is a well known antioxidant which acts by scavenging and chelating free radicals [9]. Most of the pharmacological effects of Quercetin like anti-inflammatory, immunomodulatory, anti-tumor, hepatoprotective, neuroprotective etc. are attributed to its ability to act as an antioxidant. However, clinical studies investigating these effects have not translated well to show beneficial effects mainly due to the anomalous solubility profile of the molecule. Upon oral ingestion, only 25% of the administered dose of Quercetin is bioavailable [10]. Several attempts have been undertaken to improve the bioavailability of Quercetin. However, the resultant biovailability improvement has been low [11-13]. With the use of synthetic polymers, the problem of toxicity is still unanswered. Hence, development of a suitable, biocompatible as well as bioavailable carrier for Quercetin is warranted.

Further search on the available research on Quercetin reveals that absorption of Quercetin is improved in presence of fats [14]. Hence, we decided to encapsulate Quercetin in a liposomal carrier. Liposomes are micro-particulate or colloidal carriers, usually 0.05-5.0 /~m in diameter which form spontaneously when certain lipids are hydrated in aqueous media Liposomes are composed of relatively biocompatible and biodegradable material, and they consist of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids. Drugs with widely varying lipophilicities can be encapsulated in liposomes. Liposomes can entrap both lipophilic as well as hydrophilic moieties and in doing so they protect the entrapped molecule from degradation as well as dilution in the blood stream. Moreover they offer sustained and targeted release [15]. Delivery of liposomes via the oral route has been reported in recent years. It has been shown that entrapping drug molecules into the liposomal bilayer has resulted in increased rate and amount of absorption in the gastrointestinal tract [16-17].

Parris Kidd, 1996 [18], have reported that daily intake of phosphatidylcholine has provided beneficial effects on the liver. Moreover, the liver being the major organ of metabolism, impending damage to the hepatocytes because of the myriad foods and medicines is inevitable. Oxidative stress contributes to this damage and free radical mediated injury has been indicated in liver diseases like jaundice and hepatitis [19].

Hence, we decided to formulate an oral liposomal delivery system for Quercetin with phosphatidylcholine as the lipid component and evaluate its *in-vivo* potential against CCl4 induced hepatotoxicity in rodents. Quercetin liposomes were successfully prepared by the thin film hydration technique. With an aim to maximize entrapment efficiency, the various formulation parameters affecting the loading of Quercetin into the liposomal bilayer were investigated. Effect of various parameters including the lipid composition, drug-lipid ratio and pH of the medium on the size and the encapsulation efficiency of the liposomes were studied. Particle size, drug loading efficiency and entrapment efficiency were evaluated. Transmission electron microscopy was performed to ascertain the morphology of the formed liposomes. Further, *in-vitro* dissolution studies and *ex-vivo* lipid peroxidation studies were performed to obtain the release pattern and to confirm the radical scavenging potential of the formulation. The results were further corroborated in the rodent model of CCl4 induced hepatotoxicity *in-vivo.*

MATERIALS AND METHODS

Materials

Quercetin was purchased from Fine chemicals (Mumbai, India). Phosphatidyl choline was a kind gift from Lipoid (Ludwigshafen, Germany). Cholesterol was obtained from Sigma (St. Louis, MO, USA). HPLC grade solvents were purchased from Merck (Mumbai, India). All other materials and solvents used were of analytical grade. Sprague Dawley rats used in the study were obtained from Glenmark Pharmaceuticals (Navi Mumbai, India).

Preparation of Quercetin loaded Liposomes

Multi-lamellar vesicles (MLV) liposomes consisting of mixtures of Phosphatidyl Choline and Cholesterol in different molar ratios as lipid phase were obtained by thin layer evaporation (TLE) [20]. Briefly, the lipid mixture was dissolved in 2:1 v/v of chloroform: methanol which was then removed under vacuum at 40°C, thus obtaining a thin film of dry lipid on the flask wall using a rotary flash evaporator (Superfit, India). Evaporation was continued for 2 h after the dry residue appeared, to completely remove all the traces of solvent. The film was then hydrated by adding the hydrophilic phase under vigorous mechanical shaking with a vortex mixer until vesicle formation. The liposomes were further size reduced in a probe sonicator (Oscar, Japan) to form small unilamellar vesicles. Quercetin, being lipophilic was dissolved in methanol and incorporated during film formation.

Optimization of formulation parameters for liposomes:

Initially, the effect of rotational speed of the rotovac and temperature of sonication was studied. Next, Quercetin was added to the blank liposomes. Being lipophilic Quercetin was expected to get entrapped within the lipid bilayers [21]. Hence, it was dissolved in methanol before film formation. The effect of pH was studied on the appearance of the formulation. The effect of pH on the entrapment of Quercetin is related to its pKa, regardless of the ratio of lipids employed or the amount of drug added. Hence the pH of the hydrating buffer was adjusted at values closer to the pKa of the drug and was analysed.

Further it was observed that, a stable bilayer could only accommodate certain amount of Quercetin. Further increase in the amount of Quercetin led to sedimentation. Also, the effect of these physical parameters remained constant irrespective of the ratios of lipids employed.

The above two observations were further corroborated by the values for entrapment efficiency of the formulation. Optimized formulation parameters were then used to formulate further batches. Suitable batches were then prepared to study intricately the interactions of Quercetin with the lipids and its effects on the entrapment and the particle size of the final formulation using experimental designing techniques.

Table 1: Shows the effect of speed of rotation of the rotovac, temperature of sonication and the pH of hydrating buffer on the appearance of trial batches

Rotational speed of rotovac	Result	Temp. of sonication	Result	Ph of buffer	Result
60 rpm	Bumpy film with entrapped air bubbles	Hot Temperature	Excessive clumping	pKa +1 = 7.7	Dark yellow coloured dispersion, with high sedimentation
80 rpm	Uniform Film	Room Temperature	Lumpy	pKa = 6.7	Yellow coloured suspension
100 rpm	Bumpy Film	Cold Temperature	No clumping	pKa- 1 = 5.7	Milky suspension with slight tinge of yellow.

Experimental Designing

Here, a commercially available software program was used (Design Expert, Version 7.0.2, Stat-Ease Inc and Minneapolis, MN). The experimental design chosen was Response Surface, 2- factors, 3- level factorial; 9 formulations were formulated. Run order was kept in the randomized mode to protect against the effects of time related variables and also to satisfy the statistical requirement of independence of observations. The independent factors considered were ratio of Phosphatidyl choline: Cholesterol and the amount of Quercetin. Lipid concentration was first varied to monitor the amount of drug entering the bilayer and to adjust particle size and sedimentation rate to optimum level. Quercetin concentration was then varied to achieve maximum entrapment of the drug and to adjust the drug loading and particle size. Responses chosen were the average particle size and drug entrapment efficiency.

Analysis of variance (ANOVA) and all statistical analyses were also performed using the same software. Calculation of the effects was performed; half- normal plots, response surface plots were plotted. Also ANOVA was used to treat the data, and for proper model selection. The F value was checked to see whether it is within the desired limits. The F value was calculated by comparing the treatment variance with the error variance. The 3D response surface graph is as shown in figure1.

Table 2: Shows coded and actual values of the independent variables used in the experimental design.

Phosphatidyl Choline: cholesterol (a)	Coded value	Actual Concen-tration	Amount of quercetin (b)	Coded value	Actual concen-tration(gm)
Low	-1	9:0	Low	-1	0.02
Medium	0	9:1	Medium	0	0.04
High	1	9:2	High	1	0.06

The formula of Quercetin loaded liposome was based on 3^2 factorial design where each of the two factors were considered at three levels. Thus in total, 9 batches were prepared and were observed visually for their colour and checked for odour by smell.

DSC analysis

DSC analysis was performed to ascertain the lack of potential interactions between the formulation components and to confirm the formation of liposomes. The instrument- Mettler, Toledo, DSC 821e, was calibrated with indium standard. Accurately weighed samples were placed in open, flat bottom, Aluminum sample pans. Thermograms were obtained by heating the sample at a constant rate of 10^0C/minute. A dry purge of nitrogen gas (20ml/min) was used for all runs. Samples were heated from 35^0C – 400^0C. Scans were obtained from the samples. The melting point, peak maxima, the presence and absence of endotherm peaks were observed in the DSC graphs.

Determination of Drug Entrapment Efficiency, Drug Loading and Particle Size of Quercetin Liposomes

The encapsulation efficiencies of Quercetin in liposomes were determined by an indirect method [22]. Each liposome dispersion sample was transferred into ultrafiltration tube and centrifuged at 15,000 rpm for 60 min in a refrigerated ultracentrifuge, Eltek RC 4100 D, India. After centrifugation, the supernatant was separated. The formed pellet was washed with a 1% solution of SLS in distilled water and vortexed (REMI, India) for 3 min, in order to dissolve the free Quercetin adsorbed onto the surface of the liposomes. The resulting dispersions were centrifuged for 30 min at 15,000 rpm (Eltek RC 4100 D, India). The drug content in both supernatants after centrifugation

was measured by the developed HPLC method. Quercetin content in liposomes was calculated as the difference between the total amount of the drug added to the preparation and the amount of unentrapped drug. Experiments were performed in triplicate.

For HPLC analysis, a mobile phase system comprising of methanol–water (70:30 % v/v) was utilised. 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-microlitre 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 x 250mm, 5 µ particle size). Mobile phase flow rate was 1ml/min. Quercetin was analyzed at a wavelength of 256 nm.

The drug loading (DL%) was calculated according to equation as follows:

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

The average particle size of the liposomes were calculated with a Zetasizer, Malvern Instruments, Malvern, UK) using helium-neon laser.Principle of photon correlation spectroscopy was utilized to assess mean particle size (z- Avg.) of the Quercetin loaded liposomes. Liposomal preparations were suitably diluted with distilled water in order to avoid multiscattering phenomena.

Transmision Electron Microscopy:

The morphology of the vesicles of the optimized batch was determined by a negative stain electron microscopy method in a transmission microscope (Philips CM 200). The samples were suitably diluted and contrast was improved using 2% tungstophosphoric acid. One drop of sample was placed on a copper grid coated with carbon film, dried for 3-5 min and drained on the filter paper. The grid was further dried by placing in a petriplate at room temperature, which was then loaded in the TEM and the areas were scanned for observation of vesicles [23].

***In- Vitro* Drug Release from Quercetin Liposomes:**

The dialysis bag method was applied to investigate the drug release behavior from the liposomes [24]. Liposomal suspension equivalent to 10mg Quercetin was placed in a dialysis bag. The dialysis membrane containing the sample was suspended in 200 ml medium with the help of a Rhetort stand and assembled on a magnetic stirrer. The contents were stirred 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. The pH of the dissolution medium was kept 1.2 for 2 hours using 0.1 N HCl. After 2 hours, the pH of the dissolution medium was adjusted to 7.4 with 0.1 N NaOH, buffer salts and maintained up to 20 hours. Aliquots of 2mL were withdrawn at intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 18 and 20h. They were filtered after withdrawal and the apparatus was immediately replenished with 2mL of the fresh buffer medium. The withdrawn aliquots were diluted sufficiently and 20µl solution was injected into the HPLC system for analysis, at a wavelength of 265nm.

***Ex-Vivo* Lipid Peroxidation Studies:**

Preparation of Rat Liver Homogenate: Sprague dawley rat (weighing 100-150gm, fasted overnight before experiment) was sacrificed using an overdose of anesthetic ether. The liver was quickly removed and chilled in ice cold saline. After washing with ice cold saline, the liver was homogenized in 0.15 M KCl to get 10% liver homogenate.

Methodology:

The method proposed by Ohkawa [25] was used for estimation of inhibition of lipid peroxidation.

• Fresh liver homogenate (0.2 ml) was mixed with 150 mM KCl (0.1 ml) and TRIS buffer (0.4 ml). The test samples of plain quercetin, QL and QB (0.1 ml) were then added in various concentrations.

• In vitro lipid peroxidation was initiated by addition of 0.1 ml each of $FeSO_4$ (10µM) and quercetin (100 µM).

• After incubation for 1hr at 37^{0}C, reaction was terminated by addition of TBA (Thio Barbituric acid) reagent (2ml) and boiled at 95^{0}C for 15 mins for development of colored complex.

• After cooling, the tubes were centrifuged at 4000 rpm for 10 mins. The absorbance of supernatant was determined colorimetrically at 532 nm.

• Percentage inhibition of TBA reacting substances (TBARS) formation were calculated with respect to control in which no test sample were added. The inhibition of lipid peroxidation was determined by calculating the % decrease in the formation of TBARS and IC_{50} was calculated.

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

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

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

***In-Vivo* Hepatoprotective Studies**

All studies were carried out in accordance with the principles of Laboratory Animal Care and the experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (Animal House Registration No. 25/1999/CPCSEA). Hepatoprotective activity was assessed by damaging the liver of Female-Sprague Dawley rats using CCl4 and evaluating the extent of recovery of the damaged liver by use of the test formulation [26]. Twenty four Female Sprague-Dawley rats (150-200g) were randomly divided into four groups and were fasted overnight before the experiment.

Liver damage was induced in Female Sprague-Dawley rats by administering a 1:1 (v/v) mixture of CCl4 and olive oil (1ml/kg) intraperitonealy once daily for 7 days. Group I was given Quercetin loaded liposomes (75mg/kg) by oral administration. A suspension of Quercetin in 1% c.m.c (50mg/kg) was given orally to Group II. Groups I and II received liposomal Queretin and plain Quercetin treatment for 10 days, this treatment beginning 3 days prior to the commencement of CCl4 administration. Group III served as toxicant control and received only the toxicant for the last seven days. Group IV received only olive oil intraperitoneally (1 ml/kg) daily for the last seven days. Group IV was the reference or the normal group. The degree of protection was evaluated by determining the marker enzymes SGOT, SGPT [27], Bilirubin [28] and total proteins (TP). Further, the effects of Quercetin liposomes on glutathione (GSH) [29], superoxide dismutase (SOD) [30] and catalase (CAT) [31] were estimated in liver homogenates to evaluate antioxidant activity. Histoarchitectural studies were also carried out to corroborate the *in-vivo* findings.

RESULTS AND DISCUSSION

Optimization of formulation parameters for liposomes

Three different ratios of lipids were tried. Initially, the effect of rotational speed of the rotovac and temperature of sonication was studied. Lower speeds increased the time of contact of the film with the hot water in the water bath. At higher speeds, sufficient time was not available for the lipid to form film and proper liquid to gel transitions of the lipid could not occur. Both of this may have caused uneven distribution of heat leading to formation of uneven film. Based upon the observations cited in Table 1, 80 rpm was considered optimum for formation of uniform films.

Initially sonication was performed in a bath sonicator. However, it generated lot of heat and led to the formation of clumpy formulations and the results were not reproducible. This was because the formation of the film was influenced by a number of factors like level and temperature of water, position of liposome in the bath, etc. At room temperature too, the results were not acceptable. Moreover, exposure to excess heat could catalyse the hydrolysis of lipids in the formulation [15]. Hence, probe

sonication was employed. For a sonication cycle of 10min, an on:off ratio of 1:2 secs were employed by placing the dispersion in a cold water bath. Heat generated by the sonication was dissipated by the cold water. This alternate freeze-thaw cycle yielded a uniform and non-clumpy formulation. Also, the effect of these physical parameters remained constant irrespective of the ratios of lipids employed.

Next, Quercetin was added to the blank liposomes. Being lipophilic Quercetin was expected to get entrapped within the lipid bilayers. According to Handerson Hasselbalch equation, pKa is defined as the extent to which a drug is available in the ionized form at a given pH. pH = pKa + log (ionized/unionised)

Hence, the extent of ionization is influenced by the pKa of any drug. Thus, entrapment of Quercetin is related to its pKa, regardless of the ratio of lipids employed or the amount of drug added. The pKa of Quercetin as cited in the literature was found to be 6.7. Maximum ionization (~90%) would hence occur at pH values ± one unit of the pKa value. As expected, maximum entrapment of the drug was achieved at a pH of 5.7. Hence, further batches were formed at this pH value.

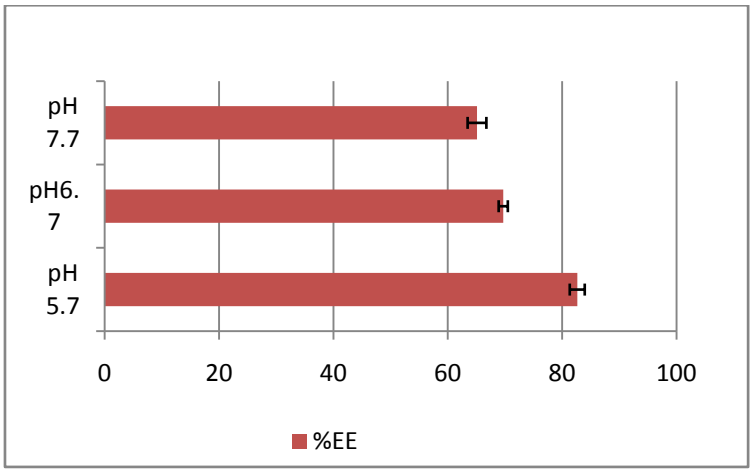


Fig. 1: Shows entrapment efficiency of liposomal formulations at different ph values (Data: Mean ± Sd, n=3)

The molecular weight of Quercetin is ~340. This factor is of importance when the amount of drug that enters the lipid bilayer needs to be considered.

The bilayer can only incorporate certain amount of drug after which the stable liposomal bilayer starts sedimenting immediately upon formation. The solubility of Quercetin in methanol also needs consideration so as to optimize the amount of drug entering the bilayer. The threshold for Quercetin was found to be 1 part of drug per 10 parts of lipid employed. The above two observations were further corroborated by the values for entrapment efficiency of the formulations.

Experimental Design

The technique of 3^2 factorial design with 2 factors at 3 different levels affecting the particle size and entrapment efficiency was considered. All experiments were carried out in random order to nullify the effects of extraneous or nuisance variables. The results of the experimental design were analyzed using Design Expert software that provided considerable useful information and reaffirmed the utility of statistical design for conducting experiments. The selected independent variables like the ratio of lipids employed and concentration of Quercetin significantly influenced the particle size and drug entrapment efficiency that is very much evident from the results in Table 2 which represents the various combinations of independent variables with its resultant effect on the dependant variable.

The experimental data were fitted into a quadratic polynomial model using the design-expert software and the equation in terms of the coded factors for the optimum particle size was found to be:

Particle Size =102.34 +144.43 * A +21.32 * B +10.90 * A * B +120.13 * A^2 +183.08 * B^2

In this case A, A^2, B^2 are significant model terms, with their P-values less than 0.05. The value of the determination coefficient R^2 was 0.9639, which implies that 96.39% of the variation in the responses was attributed to the independent variables. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 11.674 indicates adequate signal and that the model can be used to navigate the design space.

In case of the encapsulation efficiency too, experimental data were fitted into a quadratic polynomial model using the design-expert software and the equation in terms of the coded factors for the optimum particle size was found to be

Encapsulation Efficiency = +66.74 +0.99 *A+17.20 *B-8.49*A*B -9.93*A^2-2.12 * B^2

In this case, B, AB, A^2 were significant model terms. coefficient R^2 was 0.9147. Adeq Precision ratio of 13.856 indicates an adequate signal and that the model can be used to navigate the design space.

Response plots indicate that an optimum ratio of 9:1 generates particles of optimum size. Increasing or decreasing this ratio causes changes in particle size. The entrapment efficiency is also highest when ratio is 9:1.

The formation of the lipid bilayer and its fluidity is accounted by the amount of cholesterol inserted between the phosphatidylcholine molecules. Presence of cholesterol is advantageous as it makes the bilayer sufficiently flexible and also contributes to proper release of the entrapped moiety especially in case of drugs like Quercetin that interact with the lipid bilayer. [32] Highest entrapment was obtained with the batches having Phosphatidyl choline:Cholesterol ratio as 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. This can be explained by the fact that cholesterol might be replacing Quercetin in the bilayer.

Particles in the desired size range was provided by the same ratio of Phosphatidyl choline:Cholesterol. A ratio of 9:2 increased the particle size considerably. This increase in particle size might be because increased amount of lipid provides additional space for drug molecules to entrap. Although, the batches with no cholesterol yielded particles in a similar size range as compared to the ones with cholesterol in the ratio of 9:1, their entrapment efficiencies were lower.

Table 3: Shows observed values for the different levels of experimental design

Run	Block	Factor A ratio of PC: CHOL	Factor b Amount of quercetin added (GM)	Response 1 Avg. Particle size (NM)	Response 2 Entrapment efficiency (%)
1	Block 1	-1.00	1.00	533.3	77.9
2	Block 1	1.00	0.00	108.6	60.89
3	Block 1	1.00	1.00	617.3	65.02
4	Block 1	-1.00	0.00	131.7	63.14
5	Block 1	-1.00	-1.00	307	22.15
6	Block 1	0.00	1.00	281.8	82.64
7	Block 1	1.00	-1.00	542.6	43.21
8	Block 1	0.00	-1.00	259.7	57.01
9	Block 1	0.00	0.00	134.4	64.65

Response plots (Fig. 3) indicates that on increasing amount of Quercetin, entrapment efficiency was increased. This indicated that the liposomes could encapsulate 1 part of drug conveniently per 10 parts of lipid. Also, there were not much appreciable changes in particle size in light of amount of Quercetin in liposomes. This shows that entrapment of a maximum amount of drug produced little changes in particle size and all the multilamellar vesicles were formed in the range of 150-600nm.

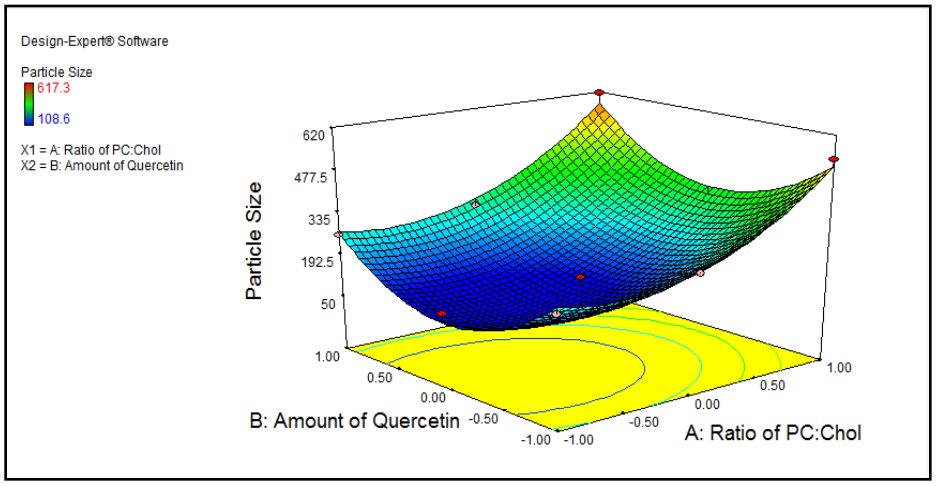


Fig. 2: Shows response surface plot for particle size

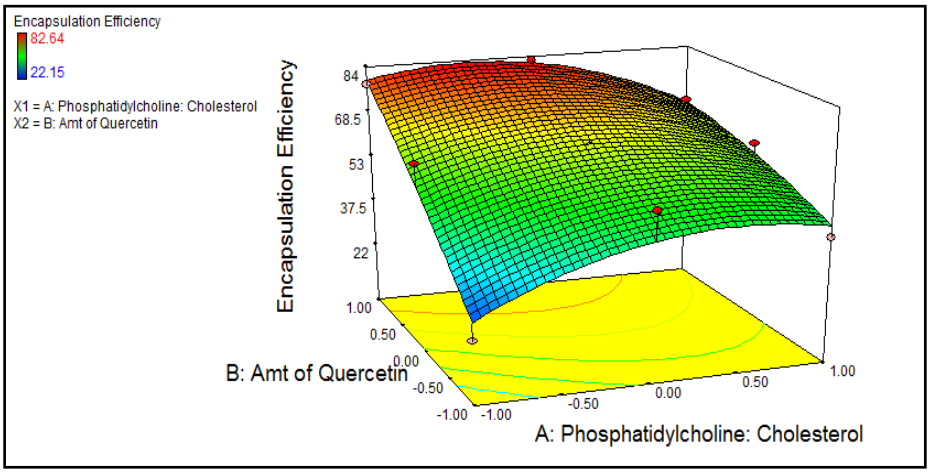


Fig. 3: Shows the response surface plot for drug entrapment efficiency

Drug Entrapment Efficiency, Drug Loading and Particle Size Of Quercetin Liposomes

Optimized preparations as predicted by the experimental designing were successfully prepared. The optimized batch gave an entrapment value of 83.733 ± 1.33 (n=3) and the mean particle size was 271 ± 32.34. A drug loading of 81.82 ± 1.30 was obtained indicating that almost 8 parts of Quercetin was entrapped per 10 parts of lipid.

Transmission Electron Microscopy

The morphology of Quercetin liposomes was as shown in fig.4 (Bar: 1 unit = 50nm). From the micrograph, it could be observed that

multilamellar vesicles with an intact bilayer membrane are formed. The particle size of the vesicles was about 270nm.

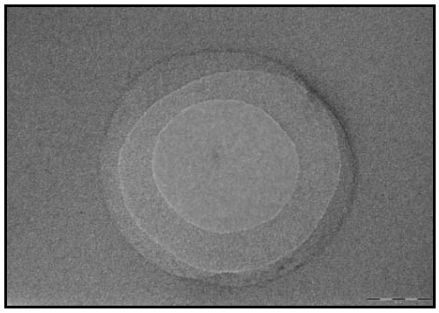


Fig. 4: Shows tem image of quercetin loaded liposomes

DSC

Thermal behavior of Quercetin, Phosphatidylcholine, Cholesterol and the physical mixture were studied using DSC. DSC thermogram of Quercetin showed an endotherm at 310.36^{0}C. Broad peaks were observed for phosphatidylcholine (~50 to 220^{0}C). For Cholesterol, the melting process took place with maximum peak at 147.10^{0}C. The thermogram of the physical mixture was almost the overlap of each individual component, except for some slight differences. DSC thermogram of the physical mixture showed the peak of cholesterol at ~147^{0}C, and integrated peak of Quercetin at ~310^{0}C. The area under the curve for the Quercetin thermogram was lesser as compared to that of Quercetin alone. This may be due to the melting of the lipid components and their interactions with Quercetin. Partial incorporation of Quercetin in the melted lipid is likely.

The DSC spectrum of the complex reveals the characteristic absence of the melting peak of Quercetin at 310^0. 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 [33]. This can be considered as indicative of drug amorphization and/or inclusion complex formation.

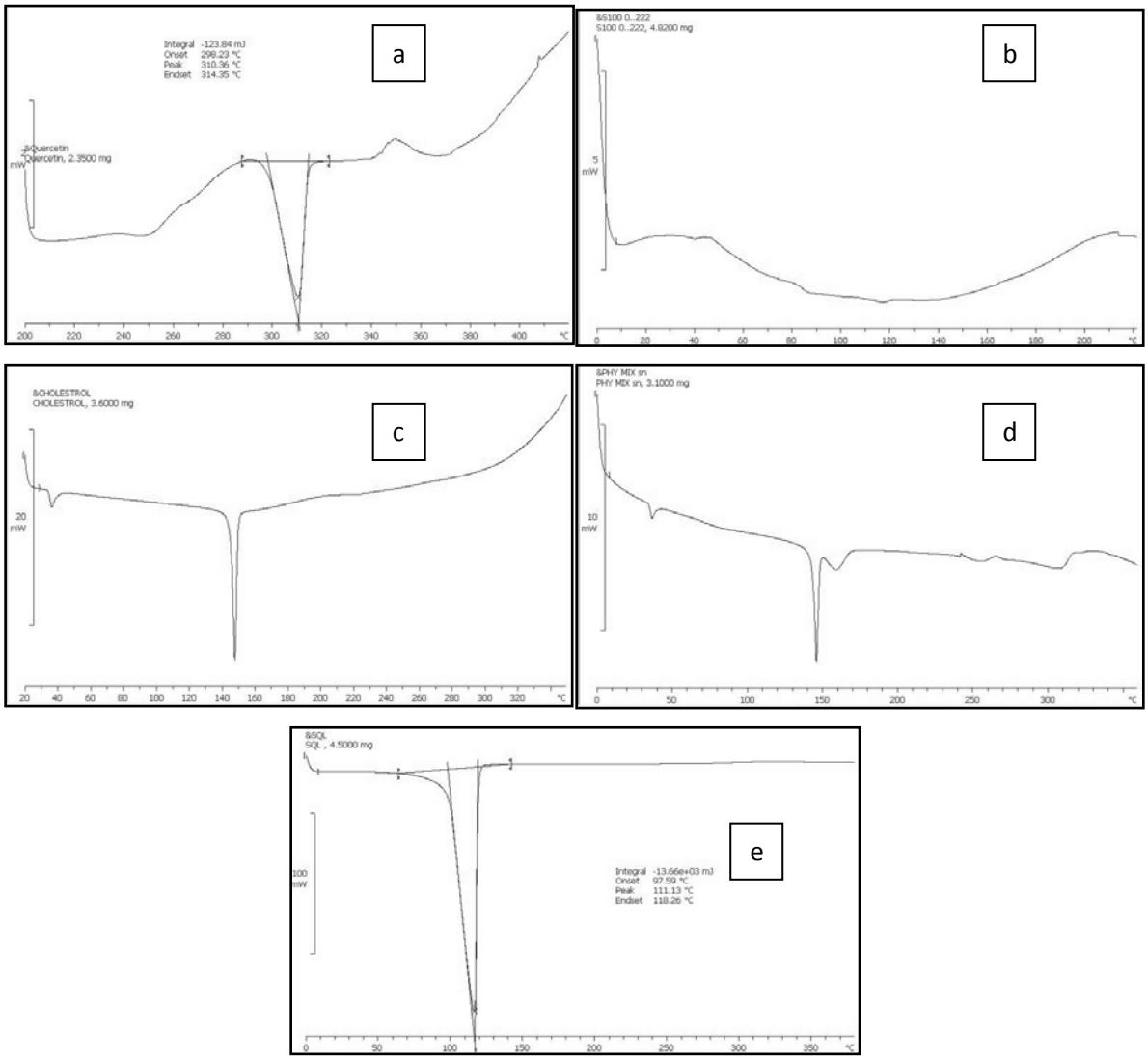


Fig. 5: DSC spectrum of a) Quercetin b) Phosphatidylcholine c) Cholesterol d) Physical mixture d) Quercetin Liposomes

***In-Vitro* Release Studies:**

The release profiles of Quercetin and Quercetin liposomes are as shown in Fig.6. The liposomes exhibited biphasic release profile. An initial burst release was observed during the first 5h followed by a continuous and slower sustained release. As expected, encapsulation of Quercetin into liposomes led to controlled release rate due to the well known reservoir effect of liposomes.

The release of Quercetin was a minimal 10% during the acidic stage i.e at pH 1.2, while upto 70% of the drug was released in a sustained manner at a pH of 7.4. Plain Quercetin showed a release pattern with

almost negligible amount of drug being released in the first 2h, an appreciable release for the first 5h followed by only a mere ~20% final release. Entrapment into liposomes has thus increased the release patern of Quercetin almost three fold.

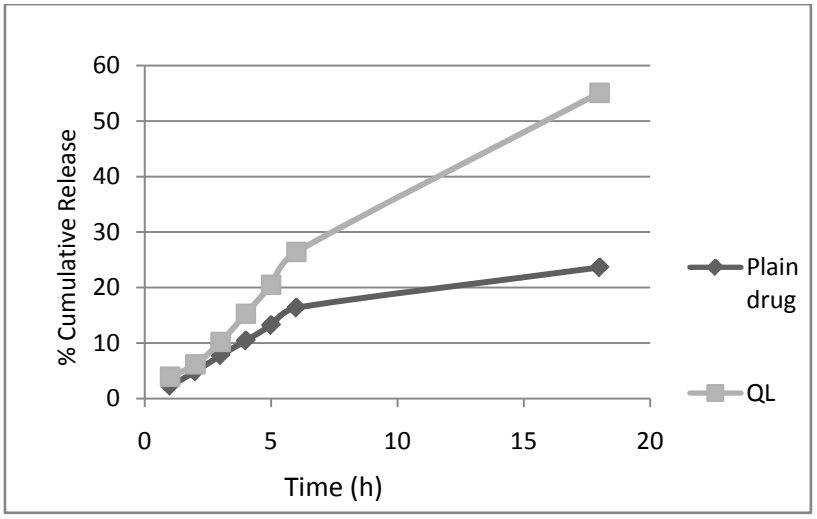


Fig. 6: Shows the *in-vitro* release profile of plain quercetin and queretin loaded liposomes

***Ex-Vivo* Lipid Peroxidation Studies:**

This test, involves reacting thiobarbituric acid with malondialdehyde produced by lipid hydroperoxide decomposition to form a red chromophore with peak absorbance at 532 nm. This colored complex results in the condensation of 2 moles of TBA and 1 mole of malondialdehyde, under the joint effect of the medium temperature and pH. The antioxidant activity of Quercetin is attributed also due to its ability to inhibit lipid peroxidation. However, encapsulation into liposomes improved this potential by many folds. This ability of Quercetin is expressed by its IC50 value, which represents the amount of Quercetin required to produce 50% inhibition of lipid peroxidation. This value for plain Quercetin was found to be of 3198.86 ± 43 µg/ml as compared to 126.94 ± 52 µg/ml for liposomes. The IC_{50} values of Quercetin loaded liposomes indicated that the anti-lipid peroxidative activity was strikingly almost 50 times more than the activity elicited by Quercetin suspension in distilled water. This is in agreement with previous findings as reported by Terao [34].

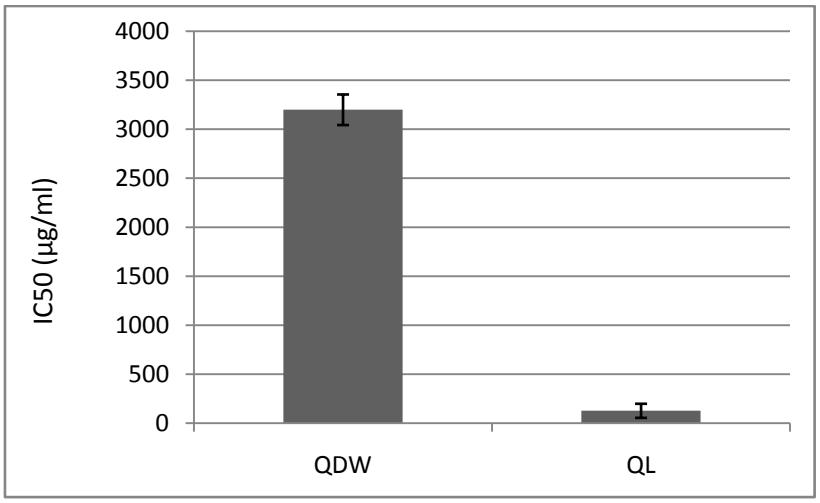


Fig. 7: Shows the results of Ex-Vivo lipid peroxidation studies – IC50 values of plain quercetin and quercetin loaded liposomes:

***In-Vivo* Hepatoprotective Studies**

The Bilirubin levels were significantly increased ($P < 0.001$) in CCl_4 treated animals when compared with the base values. Although Quercetin suspension showed significant hepatoprotective activity ($P < 0.01$), incorporation of Quercetin into lipid carriers showed improved protective effects.

Serum levels of SGPT and SGOT were significantly reduced ($P < 0.001$) by the liposomes as compared to the CCl_4 treated group. Quercetin suspension produced significant effects, however at a lower level of confidence (P < and 0.01). SOD levels were strikingly reduced in CCl_4 treated animals when compared with the normal group. Although Quercetin suspension did not significantly alter the SOD levels, at the same dose the liposome was able to produce significant increase ($P < 0.01$). The reduced levels of CAT in the CCl_4 treated animals, were significantly restored by the liposomes. Glutathione enzyme system includes glutathione, glutathione reductase, glutathione peroxidase and glutathione-S-transferase. GSH reduces toxic metabolites and oxidants and plays a significant role in maintaining the cells redox state [36]. Elevation in the levels of GSH by liposomes suggests that hepatoprotection could also be due to elevation in levels of GSH.

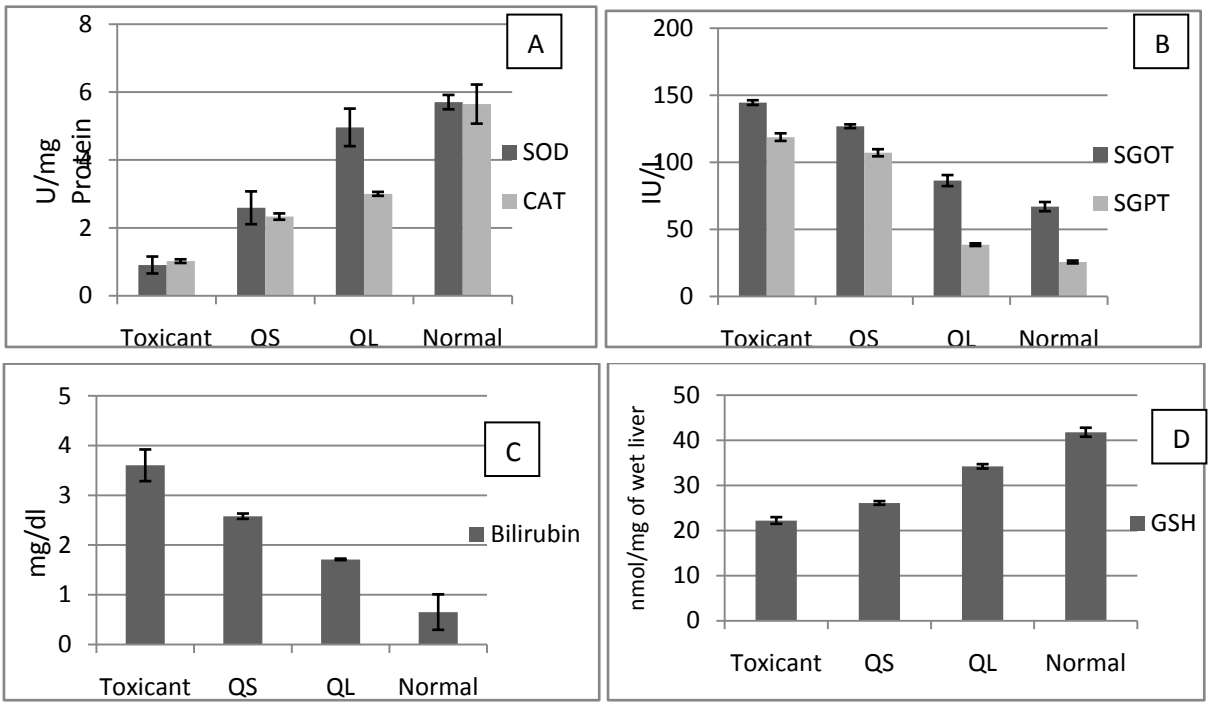
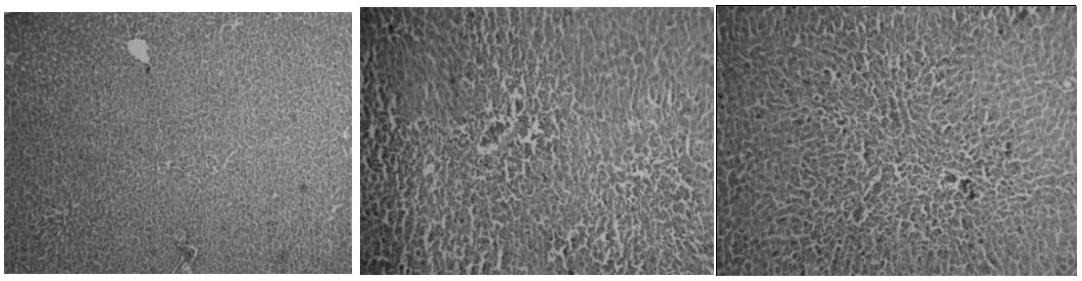


Fig. 8: Shows the effect of quercetin liposomes on a) Serum SOD and CAT, b) Serum SGOT, SGPT c) bilirubin and d) GSH levels.



A) **B)** **C)**

Fig. 9: Shows liver sections of rats treated with a) carbon Tetrachloride b) quercetin liposomes c) untreated rats stained with haematoxylin and eosin (x100)

The liver sections of CCl_4 treated animals showed centrilobular necrosis, fatty infiltration around the necrosed areas, parenchymal damage and granular degeneration towards the periphery. However, the degree of lesions was considerably mild in the groups treated with the liposome and Quercetin suspension. Regenerative foci could be distinguished in the liver sections of animals treated with Quercetin liposomes.

CONCLUSIONS

Quercetin is an antioxidant molecule found abundantly in the plant kingdom. However the molecule possesses low bioavailability. The main reason cited being the chemical structure of the molecule which hampers its bioavailability. In plants, it is found in the form of its glycosides, which are not absorbed as such by humans, leading to low systemic levels of the molecule [35]. Here, we established the improved effectiveness of the molecule by encapsulating it into a liposomal carrier. Quercetin liposomes were successfully prepared by an economically feasible method. All the formulation parameters were optimized and the optimized formulation was evaluated. The optimized formulation was found to contain phosphatidylcholine: Cholesterol in the ratio of 9:1, and it could entrap a maximum of 1:10 drug: lipid. DSC confirmed the formation of the liposome and the release studies showed sustained release pattern. The optimized formulation showed considerable oral hepatoprotective activity when compared with the plain drug in rats. The method offers a new approach to enhance the gastrointestinal absorption of Quercetin. However, in light of the present findings, the exact mechanism of improved action of Quercetin needs further studies.

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