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Research Article

NOVEL DOUBLE LOADED QUERCETIN LIPOSOMES: EVIDENCE OF SUPERIOR THERAPEUTIC POTENCY AGAINST CCL_4 INDUCED HEPATOTOXICITY – A COMPARATIVE STUDY

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ABSTRACT

Use of natural products as hepatoprotective agents is beneficial both therapeutically as well as prophylactically. However, the herbal molecules posses lower bioavailability. The principles of novel drug delivery systems when combined with herbal products can have successful outcomes. Quercetin is a herbal flavonoid molecule found to posses antioxidant activity nevertheless has lower bioavailability due to the solubility issues associated with it. The present experiment was undertaken to study the effect of novel liposomal carrier on the residential bioavailability of quercetin in terms of its antioxidant activity against CCl_4 induced hepatotoxicity. Female Sprague dawley rats were rendered cirrhotic by the intraperitoneal administration of 1:1 v/v of CCl_4: olive oil. The liver of these animals showed cell necrosis, fibrosis, and inflammatory infiltration. Histological abnormalities were accompanied by parenchymal damage and granular degeneration. These were further confirmed by estimating the levels of SOD, CAT, GSH, SGOT, SGPT and bilirubin. Results obtained indicate that quercetin in the carrier shows better amelioration of the oxidative stress elicited by the hepatotoxin, results in a cytoprotective effect, and improves the CCl_4-induced alterations in the liver structure when compared with that of free drug.

Keywords: Quercetin, liposomes, beta-cyclodextrin, hepatoprotective, carbon tetrachloride

INTRODUCTION

The liver is a vital organ liable for a number of physiological functions the prime one being metabolism of ingested molecules which would include drugs and chemicals [1]. Hence, the liver is the organ which has to bear the brunt of the many number of drugs that we consume. Moreover, many of the drugs which are corrosive in nature, cause damage to the hepatocytes and the hepatic tissues. Diseases like jaundice and hepatitis too cause damage to the liver. The importance of using natural products for therapy cannot be stressed enough [2]. However, inspite of a number of natural products available as hepatoprotective agents, their full potential has not been tapped yet. Recently, the involvement of free radicals and reactive oxygen species has been postulated in the pathogenesis of liver disorders and hence treatment with naturally available antioxidants is a viable option both therapeutically as well as prophylactically.

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid molecule distributed ubiquitously in the plant kingdom in the rinds and barks. Red wine, green tea, onions etc. are some of the rich sources of quercetin [3]. A number of pharmacological activities are associated with the molecule like gastroprotective, immunomodulatory, anti-tumor, anti-inflammatory, cardioprotective, bacteriostatic and secretory properties [4,5,6]. Many of these activities are accounted by the ability of the molecule to scavenge free radicals. Its hepatoprotective effects too seem to stem from its antioxidant activity. Inhibition of lipid peroxidation and chelation are the basic mechanisms behind antioxidant effects of quercetin [7].

However, the proposed activities have not translated well to show effects in the human body because of the low bioavailability of the molecule especially upon oral administration. 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 [8].

The recent developments in the field of novel drug delivery systems has demonstrated superior efficacy and safety in the delivery of drugs with unfavorable pharmacokinetic and physiochemical properties, as well as enhancing patient compliance. Quercetin is further known to be better absorbed in presence of lipids [9]. In the present study, liposomes were hence chosen as the nanocarrier for Quercetin aglycone. However, the entrapment of poorly water soluble drugs in the liposome's lipid bilayer is often limited in terms of the drug to lipid mass ratio. Hence, the drug was converted to a water soluble form by formulating the quercetin-betacyclodextrin inclusion complexes and these were encapsulated into the aqueous interior of the liposomes. Such drugs in cyclodextrins in liposomes improve the drug loading into the carrier and also control the invivo fate of the drug in a better manner [10].

The study was undertaken to establish the superiority of double loaded liposomes of quercetin over the Plain quercetin liposomes as well as the free form of the drug by evaluating the hepatoprotective activity attributed to its antioxidant property in rats with CCl_4 induced liver damage. The levels of various biochemical markers like super oxide dismutase (SOD), Catalase (CAT), GSH, bilirubin etc. will be assayed and the alterations will be correlated with histological changes in the liver sections.

MATERIALS AND METHODS

Drugs and chemicals

Phosphatidyl choline was obtained as a gift sample from Lipoid, Germany. Beta cyclodextrin (BCD) was a kind gift from Gangwal chemicals, Mumbai, India. Quercetin (98% pure) was purchased from Fine chemicals, Mumbai, India. Silymarin standard, epinephrine, 5,5' – dithiobis- 2-nitrobenzoic acid (DTNB) was purchased from Sigma Chemical Co., St.Louis, MO, USA. Thiobarbituric acid (TBA), reduced glutathione and nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Himedia Laboratories, Mumbai, India. All other chemicals and solvents were of analytical grade and procured locally.

Preparation of Quercetin Beta-Cyclodextrin complexes

The method proposed by Nagai et.al. was followed for the preparation of quercetin beta-cyclodextrin complexes [11]. Briefly, Quercetin and BCD in 1:1 molar ratio were accurately weighed and dissolved in distilled water. To this aqueous solution further, dilute ammonia was added to dissolve the quercetin. The whole solution was stirred on magnetic stirrer for 2h till a clear solution resulted. The solution was frozen overnight and then lyophilized. The free flowing solid was then passed through sieve no.60 and stored in a dessicator.

Preparation of Quercetin Liposomes (QL)

Liposomes were prepared by the classic hand-shaking method. The lipids i.e phospholipid, cholesterol (in different ratios) and the drug were dissolved in suitable organic solvents. The solutions were then

taken in a round bottom flask (RBF). The solvent was evaporated and a thin film was formed using a rotary flash evaporator (Superfit, India). The film was hydrated using buffer solution (0.05 M) and hand shaken till the film sticking on the sides were completely removed and a milky suspension containing multilamellar vesicles result [12]. The liposomes were further size reduced in a probe sonicator (Oscar, Japan) to form small unilamellar vesicles.

Preparation of Novel Double Loaded Quercetin Liposomes (QB)

Essentially, the same method stated as above was used to prepare liposomes. However, while hydrating the lipid film with buffer, the Quercetin BCD complexes were dissolved in the buffer and then incorporated in to the aqueous phase of the liposomes. Thus the drug was "double" loaded into the lipid bilayers as well as the aqueous compartment of the liposomes. The suspension form of the formulation was stored in air tight container at 2-8^0 for further use.

Preparation of test and reference solutions

Both silymarin and quercetin (QS) were administered as an aqueous solution of 1% c.m.c. The suspended form of the liposomes were used as such.

Experimental Animals

Female Sprague Dawley rats (150-200g) were used for the study. They were housed in clean polypropylene cages in groups of five, under standard conditions of temperature (20-25^{0}C) and light (12h light/12h dark cycle) and fed with a standard diet and water ad libitum. All animals were handled with humane care. Experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (Animal House Registration No. 25/1999/CPCSEA).

Dosing

The rats were divided into seven groups of five rats each. They were allowed to acclimatize for a week and then dosed after overnight fasting.

Group I – served as normal control and received olive oil (1 ml/kg, i.p.) daily for 7 days.
Group II – served as toxicant control and received 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) daily for 7 days.
Group III – received Standard Silymarin (50 mg/kg, p.o.) daily for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.
Group IV – received plain Quercetin Suspension - QS (50 mg/kg, p.o.) daily for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.
Group V – received QL (75 mg/kg, p.o.) for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.
Group VI – received QB50 (50 mg/kg, p.o.) for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.
Group VII – received QB75 (75 mg/kg, p.o.) for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.
Animals were humanely killed by light ether anaesthesia 24 h after the last dosing [13,14].

Blood was collected by puncturing the retro-orbital plexus as well as by cardiac puncture and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation at 4500 r.p.m at 30^{0}C for 15 min and used for the assay of marker enzymes viz., SGOT(Serum glutamic oxaloacetic transaminase) , SGPT(Serum glutamic pyruvic transaminase) and Bilirubin. The livers were dissected out immediately, washed with ice-cold saline and 10% homogenates in 1.15% KCl were prepared. The homogenates were centrifuged at 10,000 r.p.m for 10 min, and the supernatants were used for the assays of SOD(Superoxide dismutase), CAT(Catalase) and GSH.

Marker Enzymes

GSH estimation in the liver homogenate was performed using the DTNB (5,5'-Dithiobis(2-nitrobenzoic acid) method [15]. The absorbance of the processed liver tissue was read at 412nm.

The method by Sun and Zigman was utilized for the estimation of SOD, wherein the content of the oxidized product adrenochrome was related to the SOD content by its spectrophotometric determination at 320 nm [16].

CAT was assayed by the method of Clairborne, which involves relating the CAT activity (in terms of number of units of CAT) to the breakdown of H_2O_2 per unit time, measured spectophotometrically at 240nm, where 1U of CAT = mmol H_2O_2consumed/min/mg protein. [17].

Biochemical Parameters

The marker enzymes viz. SGOT, SGPT and Bilirubin were estimated in the serum using diagnostic kits (Span Diagnostics). Bilirubin estimation is based on the method of Malloy and Evelyn wherein azobilirubin, a red-purple colored precipitate formed by the coupling reaction of bilirubin with diazotized sulfanilic acid in acidic medium. The intensity of the purple color is measured spectrophotometrically at 540nm [18]. SGOT and SGPT were estimated according to the method of Reitman and Frankel, which is an end-point colorimetric method for the estimation of enzyme activity determined spectrophotometrically at 505nm [19].

Histopathological Studies

After sacrificing the animals, the livers were immediately removed, washed with cold saline, sectioned and fixed in 10% formalin. The sections, after dehydration, were stained with haematoxylin and eosin. They were observed under a microscope for histopathological changes in liver architecture and photographed.

Statistical Analysis

The data are expressed as mean ± standard error mean (S.E.M.). Results were statistically analysed using one-way ANOVA, followed by the Tukey–Kramer test for individual comparisons. $P < 0.05$ were considered to be significant.

RESULTS

The effects of quercetin liposomes and double loaded liposomes on the marker enzymes and biochemical parameters are summarized in tables I and II respectively.

Table 1: Effect of Quercetin doule loaded liposomes on marker enzymes viz. SGOT, SGPT, Total Bilirubin.

Marker Enzymes	SGOT (IU/L)	SGPT (IU/L)	BILIRUBIN (TOTAL) mg/dl
Group I Normal Control Olive oil (1ml/kg)	66.958 ± 3.4370	25.677 ± 1.046	0.6502 ± 0.3570
Group II Toxicant Control 1:1 CCl_4 : Olive oil (1ml/kg)	144.514 ± 1.765^a	118.770 ± 2.828^a	3.6023 ± 0.3183^a
Group III Silymarin (50mg/kg)	90.266 ± 3.729^c	57.316 ± 0.4225^c	2.1529 ± 0.1076^c
Group IV QS (50mg/kg)	126.890 ± 1.398^b	107.117 ± 2.642^b	2.5784 ± 0.0536^b
Group V QL (75mg/kg)	86.361 ± 4.129^c	38.586 ± 0.997^c	1.7100 ± 0.0152^c
Group VI QB50 (50mg/kg)	106.279 ± 2.347^c	81.656 ± 1.283^c	2.2911 ± 0.0963^c
Group VII QB75 (75mg/kg)	65.627 ± 1.571^c	35.911 ± 1.230^c	1.0705 ± 0.0765^c

Values are mean ± SEM; N = 5 in each group.

[a]P value < 0.001 when toxicant control compared with normal control.
[b]P value < 0.01 when experimental groups compared with toxicant control.
[c]P value < 0.001 when experimental groups compared with toxicant control.

Table 2: Effect of Quercetin doule loaded liposomes on biochemical parameters viz. SOD, CAT, GSH.

Biochemical Parameters	SOD U/mg protein	CAT U/mg protein	GSH nmol/mg of wet liver
Group I Normal Control Olive oil (1ml/kg)	5.7054 ± 0.2111	5.6469 ± 0.5757	41.77 ± 0.9961
Group II Toxicant Control 1:1 CCl_4 : Olive oil (1ml/kg)	0.9070 ± 0.2495[a]	1.0209 ± 0.0541[a]	22.21 ± 0.7429[a]
Group III Silymarin (50mg/kg)	4.9342 ± 0.4980[b]	2.9383 ± 0.0488[c]	35.22 ± 0.7377[c]
Group IV QS (50mg/kg)	2.5992 ± 0.4832	2.3337 ± 0.0931[c]	26.10 ± 0.4042[b]
Group V QL (75mg/kg)	4.9612 ± 0.5553[b]	3.0030 ± 0.0582[c]	34.21 ± 0.4879[c]
Group VI QB50 (50mg/kg)	4.5390 ± 0.7156[b]	2.6494 ± 0.0815[c]	31.04 ± 0.9585[c]
Group VII QB75 (75mg/kg)	5.6475 ± 0.0792[c]	3.4824 ± 0.0901[c]	38.25 ± 0.3897[c]

Values are mean ± SEM; N = 5 in each group.
[a]P value < 0.001 when toxicant control compared with normal control.
[b]P value < 0.01 when experimental groups compared with toxicant control.
[c]P value < 0.001 when experimental groups compared with toxicant control.

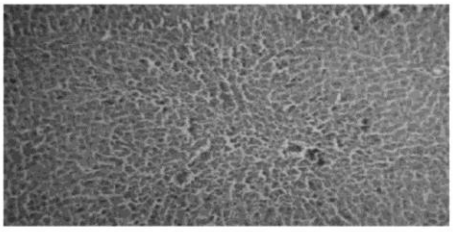

Fig 1: Liver section of normal rats stained with haematoxylin and eosin (x100)

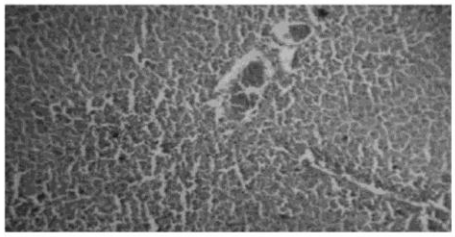

Fig 2: Liver section of rats treated with carbon tetrachloride and QB (75mg/kg) stained with haematoxylin and eosin (x100)

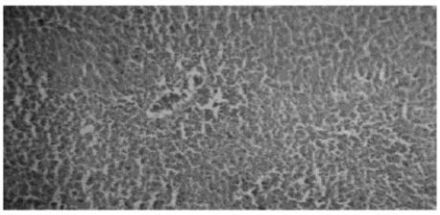

Fig 3: Liver section of rats treated with carbon tetrachloride and QL (75mg/kg) stained with haematoxylin and eosin (x100)

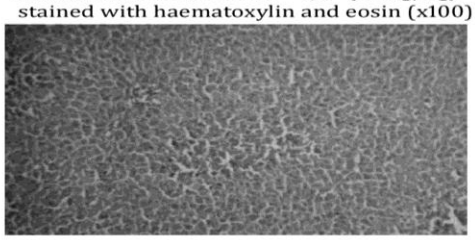

Fig 4: Liver section of rats treated with carbon tetrachloride and QB (50mg/kg) stained with haematoxylin and eosin (x100)

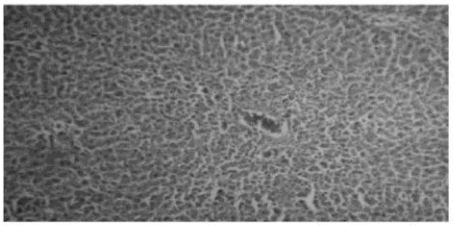

Fig 5: Liver section of rats treated with carbon tetrachloride and Silymarin(75mg/kg) stained with haematoxylin and eosin (x100)

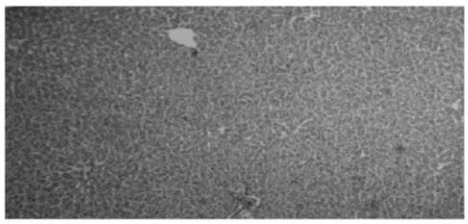

Fig 6: Liver section of rats treated with carbon tetrachloride stained with haematoxylin and eosin (x100)

Marker Enzymes

The Bilirubin levels were significantly increased ($P < 0.001$) in CCl_4 treated animals when compared with the base values. Although QS showed significant activity ($P < 0.01$), its incorporation into lipid carriers showed better protective effects and were comparable with those of the standard silymarin. While with QB75 levels almost equivalent to that of the normal were obtained, the effects at lower dose of QB50 was equivalent to plain quercetin. QL too showed activity comparable to that of the standard, however was less effective than QB75.

Serum levels of SGPT were significantly reduced ($P < 0.001$) by silymarin, QS, QB and QL as compared to the CCl_4 treated group. QB75 restored the levels of SGPT enzymes almost equivalent to the base values. SGOT levels too were restored to normal by QB75. While QL and QB50 showed effects comparable to the standard, QS was significant at a lower level of confidence ($P < 0.01$). The same pattern of activity with QB75 showing maximum protective effects were established for both SGOT and SGPT.

Biochemical Parameters

SOD levels were strikingly reduced in CCl_4 treated animals when compared with the normal group. Although QS did not significantly alter the SOD levels, at the same dose QB was able to produce significant increase ($P < 0.01$). QL showed effects similar to that of the standard, while with QB75, levels equivalent to normal was established.

The reduced levels of CAT in the CCl_4 treated animals, were significantly restored by all the treatment groups ($P < 0.001$), with QB75 showing the maximum efficacy.

Histoarchitectural Studies

Paramount differences were observed in the liver sections of normal group and that of CCl_4 treated animals. 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 QB and QL. The degree of fatty infiltration and parenchymal damage were reduced in a dose dependant manner by QB. The hepatic architecture of the animals treated with QL was also sufficiently protective in nature. Regenerative foci could be distinguished in the liver sections of animals treated with QB75, QL and silymarin.

DISCUSSION

The concept of drug in cyclodextrin in liposomes was recently reported in 1994 [10]. This delivery system was especially suitable for the chosen molecule quercetin because of the structural and physiological properties of this molecule.

Quercetin is present in plants in the form of its glycoside namely its rhamnoside i.e quercetin-3-O-rhamnoglucoside. This conjugate has a lower value of partition coefficient (log octanol/water) namely 0.37 ± 0.06 than the aglycone form of quercetin namely 1.2 ± 0.1. This means that the rhamnoside is hydrophilic and conversion to its hydrophobic aglycone form by hydrolysis is essential for its absorption and hence its activity [20]. The aglycone form however is lipophilic. This limits the degree to which it can be dissolved in the physiological fluids and hence hampers the extent of the molecules' bioavailability.

When formulated alone, because of its lipid solubility, quercetin selectively gets trapped in the lipid bilayers of the liposome [21]. This leaves the aqueous compartment empty. Hence, drug in its virgin form is entrapped in the lipid bilayers of the liposome while a water soluble cyclodextrin inclusion complex of the drug is encapsulated in the aqueous chamber within the liposomes. Thus the liposomes were "double" loaded.

The antioxidant activity of quercetin primarily stems from the fact that it is able to scavenge free radicals. Hence, CCl_4 induced hepatotoxicity was the experiment of choice. CCl_4 is a potent toxicant and causes centrilobular necrosis, hepatocellular degeneration and changes in the levels of some enzymes. Here, damage similar to that produced by free radicals is seen, as a result of conversion of CCl_4 in presence of oxygen to the trichloromethylperoxy free radical. This radical then initiates an autocatalytic chain reaction which eventually causes cell damage primarily by lipid peroxidation and rapid breakdown of structure and function of endoplasmic reticulum of the liver [22]. This in turn is reflected by the altered levels of serum enzymes like bilirubin, SGOT, SGPT. Damage to the liver cells causes rise in the concentration of these enzymes in the blood stream. This is because these enzymes are primarily cytoplasmic in origin and damage to the hepatocytes causes leakage into the blood stream. In conformation, the toxicant group demonstrated highest levels of these marker enzymes indicating maximal damage to the hepatocytes. Pretreatment with the standard drug and with QL and QB caused reduction in the leakage of these enzymes into the blood stream.

The levels of SOD and CAT too were restored to normal levels suggesting that one of the probable mechanisms of hepatoprotection could be the restoration of enzymatic levels. The restored SOD catalyses conversion of the superoxide anion to hydrogen peroxide and is further reduced to give water. CAT catalyses the conversion of hydrogen peroxide to water. Thus, the increase in levels of these two enzymes further counteracts the free radicals.

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 [23]. Elevation in the levels of GSH by QL, QB suggests that hepatoprotection could also be due to elevation in levels of GSH.

The results from the observation of histological slides showed similar results. The necrosis and fatty infiltration caused by CCl_4 was significantly reversed by QB and QL. Also, the levels of total protein content in rats treated with QB and QL was higher. This indicated that the process of regeneration of liver cells was accelerated; which is confirmed by the extent of recovery of the liver tissue treated with QB and QL.

Quercetin is a potent flavonoid with antioxidant effects. In the present study, an attempt was made to improve the residential bioavailability of the molecule by formulating it as liposomes. The liposomes were further quality improved by double loading them with cyclodextrin complexes of the drug.. Also, double the amount of drug could be incorporated in the same amount of carrier leaving the benefits of the carrier unhampered. Such a double loaded carrier would also produce enhanced effects due to immediate release from cyclodextrin complex upon contact with the physiological fluid and then sustained effects would be produced as a result of slow release from the lipid bilayers. As postulated, results indicate that at similar doses, QB fares better than the freely suspended form of drug as well as plain liposomal formulation.

In conclusion, the hepatoprotective effect of QB against CCl_4-induced hepatotoxicity in rats appears to be due to the restoration of enzyme levels. Furthermore, it may be stated that Quercetin when complexed with the lipid carrier and double loaded into liposomes, showed sustained and enhanced effects.

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