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PREPARATION, CHARACTERIZATION AND EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF AN INTRAVENOUS LIPOSOMAL FORMULATION OF BIS-DEMETHOXY CURCUMIN ANALOGUE (BDMCA)

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ABSTRACT

The aim of study was to prepare small unilamellar vesicles (SUVs) incorporating BDMCA that can injected by intravenous route and further, evaluate hepatoprotective activity of the formulation. SUV liposomes were prepared using thin film hydration followed by sonication method. Soya lecithin was used as lipid and stearyl amine was used as cationic charge inducer. In the preparation of liposomes, process and formulation parameters were standardized. After preparation SUVs were characterized for physicochemical properties, particle size, zetapotential, percent drug entrapment, in vitro drug release and the drug-polymer interaction. The sustenance of drug release into the plasma after intravenous BDMCA SUV administration was determined. Hepatoprotective activity was evaluated in CCl_4 treated rats. The liposomal formulations were successfully prepared using thin film hydration followed by sonication method. The desired encapsulation was achieved by increase in the area of the lipid film formed. The size of SUVs obtained was 327 nm. FTIR results indicate there was no interaction between lipid and drug. In vitro release data showed that the release was sustained for 10 days in vitro and could be described as diffusion-controlled. The liposomal formulations were able to sustain the release of drug in vivo also. Liposomal formulations showed better hepatoprotective activity to the drug compared to its solution form.

KEY WORDS: *BDMCA, liposomes, small unilamellar vesicles, hepatoprotective activity.*

INTRODUCTION

Curcumin, a phenolic compound from the plant Curcuma longa L., has shown a wide-spectrum of chemopreventive, antioxidant, antitumor and hepatoprotective properties. However, curcumin demonstrated poor systemic delivery. Data from several studies indicated that its poor solubility, very low GIT dissolution rate, low absorption, and its extensive systemic metabolism are the reasons for its delivery problems and lack of clinical success [1].

Structural variations in any lead compound are important for its physiological activity, especially if these affect its receptor binding interactions. Structural variations also alter its pharmacokinetics. Extensive structure activity relationship studies have been carried out on the curcumin molecule, and a large number of synthetic analogues are

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known [2]. BDMCA [1,7- bis(2-hydroxyphenyl)-1,6-heptadiene-3,5-dione] is a novel curcumin analogue. BDMCA (bis-demethoxy Curcumin analogue) has demonstrated many activities of Curcumin [3, 4, 5]. In most of the cases it showed more effect than the Curcumin [5, 6, 7, 8, 9].

We aimed to evaluate its hepatoprotective activity after administering it as a liposomal formulation and compare it with i.v. solution form. In this study, we investigated liposomal delivery approach for BDMCA (Structure 1) for its hepatoprotection

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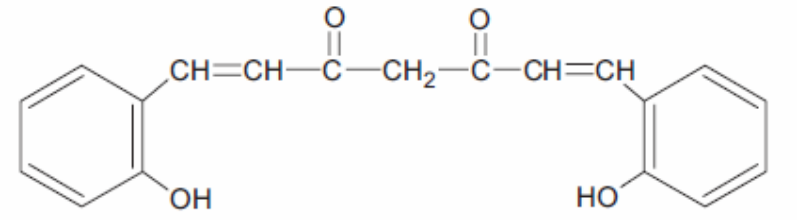

Structure 1. Chemical Structure of BDMCA

The liver is one of the most significant target for toxic substances and represents the main site for detoxification in the organism. Liver inflammation or hepatitis and liver cancer are commonly found diseases are leading causes of death. When hepatitis occurs, it causes the retention of toxic chemicals in the body. Prolong hepatitis will lead to fibrosis, cirrhosis, and liver cancer. In many of these diseases, kupffer cells and sinusoidal cells of liver are involved [10]. Interestingly, these cells can avidly take up particles from systemic circulation [11, 12]. Targeting of drugs to the intracellular space can lead to highest level of the drug that can be used in the therapy and thus there can be an increase in the effectiveness in therapeutic activity several fold. Therefore, this study developed a SUV liposomal formulation of BDMCA that can lead to enhanced intracellular levels of the drug in the liver cells thereby may demonstrate more therapeutic effectiveness. This liposomal formulation can also result in sustained release of the drug in the plasma. Keeping in view, the promise held by BDMCA as a therapeutically active agent, it is pertinent to develop a new formulation of BDMCA, which can enhance its therapeutic activity. The first objective of this study was to prepare and characterize the SUV formulation of BDMCA.

The second objective of this investigation was to evaluate the sustained release ofthe drug in the plasma and hepatoprotective activityafter intravenous administration of the developed SUV formulation containing BDMCA and compare with that of an *i.v.* solution formulation.

MATERIALS AND METHODS

MATERIALS

All the chemicals used were purchased from Finar chemicals (Ahmedabad, India), Qualigen Fine chemicals (Mumbai, India) and Merck Specialities (New Delhi, India). Soya Lecithin was purchased from Hi-media (Mumbai, India). Stearyl amine was purchased from Sigma Aldrich (Mumbai, India). Male Wistar rats (150-200gm each) purchased from Mahaveer Enterprises (Hyderabad, India) were used in this preclinical investigation. SGOT and SGPT kits were purchased from Coral Clinical Systems, Goa, India. BDMCA was synthesized and purified according to previously published reports [13, 14].

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PREPARATION OF BDMCA LIPOSOMES AND OPTIMIZATION OF THE PROCEDURE OF PREPARATION

Neutral and positive charged liposomes of BDMCA were prepared. Multi-lamellar vesicles (MLVs) consisting of BDMCA were prepared using the thin film hydration method using different formula (Table 1).

Briefly, exemplified by one batch of liposomal preparation, accurate quantities of BDMCA (20 mg), soya lecithin (120

mg) and cholesterol (25 mg) were dissolved in 10ml chloroform in a round bottom flask (Formula F9). A thin layer of lipid containing drug was allowed to form on the walls of the flask by evaporating chloroform under reduced pressure using a rotary evaporator (Laborota 4000, Heidolph, Germany) at a temperature 60^0C and 90 rpm. Residual chloroform was evaporated overnight by storing the thin film in a vacuum desiccator. To form MLVs, this layer was hydrated with 10 ml of normal saline, while vortexing the flask, which was maintained at a temperature 70^0C and 90 rpm for 1 hr. The liposomal suspension was then centrifuged at 5000 rpm for 30 min to separate the free drug and then the pellet of MLVs was resuspended in normal saline. The resulting suspension was subjected for sonication for 15min (3min each cycle, 5cycles, 150V/T probe) using a ultra-homogenizer (Biologics inc., USA) to get small unilamellar vesicles (SUVs). For the preparation of positive charged liposomes a positive charge inducer stearyl amine was added to the initial organic phase. In this preparation, stearyl amine (10 % w/w of lipid) was dissolved in 2ml of methanol and mix to 8 ml of chloroform containing BDMCA. The contents that were present in the liposomal suspension were calculated by subtracting the amount determined in the layer from total quantity taken. From our preliminary data, BDMCA encapsulation in the liposomes using the standard procedures was very less. To enhance the encapsulation, we have optimized the surface area occupied by phospholipids and drug by adding glass beads in the round bottom flask and then MLVs were prepared. MLVs thus prepared here used to prepare SUVs. The increase in encapsulation with increase in surface area was also investigated. The net surface area was increased with addition of increased number of glass beads.

ESTIMATION OF DRUG AND THE LIPIDS IN THE LIPOSOMAL FORMULATION

The drug content was estimated from calibration curve of BDMCA in chloroform containing lipid and cholesterol against blank (with out drug) at 425 nm using a UV-Visible spectrophotometer. The content of phospholipids was estimated by Stewart assay [15]. This colorimetric method is based on the formation of a colored complex between phospholipids and ammonium ferrothiocyanate reagent. Calibration graph of lipid in chloroform was obtained for amount ranging to 0.3 to 0.8 mg, analyzed against blank (with out lipid) at 485 nm using a UV-Visible spectrophotometer (Elico, SL 164 Double beam, Hyderabad). To estimate the lipid content, the layer was dissolved in 20 ml of chloroform. From that 1 ml was taken into a separate test tube and the solvent was evaporated. Then the lipid content was estimated. The content of cholesterol was estimated by colorimetric assay. This colorimetric method is based on the formation of a colored complex due to reaction between freshly prepared acetic anhydride-concentrated sulphuric acid mixture (20:1) and cholesterol. Calibration curve was plotted by taking known quantities of cholesterol and analyzed against blank (with out cholesterol) at 680 nm using a UV-Visible spectrophotometer. To estimate the cholesterol content, the layer was dissolved in 20 ml of chloroform. From that take 1 ml into a separate test tube and the solvent was evaporated. Then the cholesterol content was estimated by the above method.

IN VITRO CHARACTERIZATION OF BDMCA SUV LIPOSOMES PARTICLE SIZE AND ZETA POTENTIAL OF LIPOSOMES

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The mean size and polydispersity index of the size distribution and zeta potential of SLN was determined by photon correlation spectroscopy (PCS) using Zetasizer 3000 HAS (Malvern Instruments, Malvern, UK). The liposomal dispersions were diluted 1:1000 with the aqueous phase of the formulation to get a suitable kilo counts per second (kcps). Analysis was performed at 25°C with an angle of detection of 90°. Each value reported is the average of three measurements. The polydispersity index measures the size distribution of the liposomal (SUVs) population.

DRUG ENTRAPMENT STUDIES

The percentage drug entrapped (PDE) was determined by Ultra-centrifugation. The liposomal formulations were subjected for ultracentrifugation (ultra Centrifuge – Remi laboratories, Mumbai, India) at 11000 rpm for 30 min in an ultracentrifuge in order to separate the entrapped drug from the free drug. Then the clear supernatant was separated and analyzed for drug content after appropriate dilution by UV-Visible Spectrophotometer. This indicates amount of free drug. The liposomal pellet was redispersed in chloroform and analyzed for drug content after appropriate dilution by UV-Visible Spectrophotometer. This indicates amount of drug entrapped. The entrapment capacity of liposomes was calculated as follows

$$PDE = [(T-C)/T] \times 100$$

Where T is the total amount of drug that is detected both in the supernatant and sediment, and

C is the amount of drug detected only in the supernatant.

IN VITRO DRUG RELEASE STUDIES

In vitro release studies were performed using dialysis membrane method. An aliquot of SUV formulation was placed inside a dialysis membrane (MWCO: 25,000) immersed in aqueous buffer of volume 100 ml (PBS). At predetermined time points the dialysate was sampled and the amount of BDMCA was determined using a UV-Vis spectrophotometer as mentioned in the previous section. Drug release was monitored until no more drug was released for 30 min.

FOURIER TRANSFORM INFRARED (FTIR) STUDIES

BDMCA, soya lecithin, placebo liposomes and BDMCA loaded liposomes were subjected to FTIR analysis so as to predict if there is any interaction is possible between the drug and the lipids.

***IN VIVO* DRUG RELEASE AND HEPATOPROTECTIVE ACTIVITY OF THE FORMULATION**

In Vivo drug release was investigated in male wistar rats. All the animal experiments were conducted according to the rules and guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India. The study was approved by Institutional Animal Ethical Committee of Vaagdevi College of Pharmacy, Warangal, registered under CPCSEA, India (Registration No: 1047/ac/07/CPCSEA). The rats were acclimatised with 12 hour dark and 12 hour light cycle at a temperature of 20º C at a humidity of 60% and were fed on standard diet for 10 days prior to the commencement of the experiment. The conditions continued during the next 10 days of experimentation. The rats were divided into 3 groups, I, II and III. Four rats were used in each group. To group I, 1ml neutral liposomal formulation (SUVs) was injected, to group II, 1ml of positive charged liposomal formulation (SUVs) was injected and to group III, 1ml of drug dissolved in PEG 400 (8mg of drug in 1ml of PEG 400) was injected. This is the equivalent dose administered in the form of liposomes. All the injections were made by intravenous route. Blood samples after

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administration of the formulations were collected at 0.5, 1, 2, 3, 6, 12, 24 hours and 3, 6 and 9 days. At the end of 9 days liver samples were also collected for the estimation of the drug. The drug was extracted from the plasma by using ethyl acetate. BDMCA in these samples was estimated using a HPLC (Cyberlab, USA) at 230nm. In case of liver, the tissue was crushed and then the drug was extracted. The column used was C_{18} ODS column and the size of silica used in this column is 5µm and the dimensions of the column are 4.6 × 250mm. A HPLC standard curve for the drug in the plasma and the liver was generated. The mobile phase consisted of methanol: water at a ratio of 80: 20.

HEPATOPROTECTIVE ACTIVITY

Hepatoprotective activity was used as the pharmacodynamic end point. Hepatoprotective activity was determined for *i.v.* solution as well as liposomal administration. Male rats weighing around 150-200 gms was used in this study. For this experiment, animals were divided into 6 different groups. Group I were normal control, received 1ml normal saline daily. Group II receives CCl_4 regularly and it served as toxic control. Group II received CCl_4 at a dose of 0.7ml/kg by intraperitonial route on the 3rd, 6th and 9th day consisting of mixture of CCl_4 and olive oil (25:75). Group III received 1ml of neutral BDMCA liposomal formulation one time in the beginning of the experiment via i.v. route. Group IV received 1ml of positive BDMCA liposomal formulation one time in the beginning of the experiment via i.v. route. Group V received BDMCA dissolved in PEG 400 solution at a dose 8mg by intravenous route daily. Group VI received ethanolic suspension of curcumin by oral route at a dose 100mg/kg daily. This group was treated as standard. All groups received CCl_4 at 3, 6 and 9th day of the study except normal control. Hepatoprotective activity was quantified by the activity of SGOT (serum glutamate oxaloacetate transaminase), SGPT (serum glutamate pyruvate transaminase) levels and histological studies following a previously published report [16].

RESULTS AND DISCUSSION

Preparation and Characterization of liposomes

The liposomal formulations were successfully prepared. For optimization purpose, different liposomal formulations were prepared (Table 1) and these contained different drug lipid ratios. F9 formulation demonstrated higher drug content among all the MLV suspension. However, this percent is still lower. Only 21% of drug was present in this liposomal suspension. An attempt to increase the surface area of the phospholipid layer using small glass beads into the process of formation of thin layer was then investigated. Formulations were prepared after incorporation of glass beads in the technique. Results clearly indicate that increase in the surface area using glass beads increased the percent drug entrapment (Table 2). We observed highest PDE for formulation F13. And this formulation was selected to prepare SUV formulations of neutral and positive charge that were injected into the rats.

Table 1: Effect of drug-lipid ratio on percentage of drug in liposomal suspension

Formulation	D:PC:CH Ratio/ Amounts (mg)	PLLS*	PCLS*	PDLS*	SIZE(µm)**
F1	1.0:10:0/10:100:0	75±3.5	----	7.2±0.9	5.86±2.356
F2	2.5:10:0/25:100:0	72±3	----	5.4±0.5	5.99±1.981
F3	0.5:10:2.5/5:100:25	71.15±2.7	15.87±2.1	6.5±0.7	5.12±1.942

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F4	1.0:10:2.5/10:100:25	75.64±3.9	17.14±1.9	7.9±1.2	5.54±2.320
F5	1.5:10:2.5/15:100:25	78.12±3.2	19.86±1.23	17.06±1.4	4.37±1.940
F6	2.0:10:2.5/20:100:25	87.37±4.9	19.99±0.9	19.9±1.5	4.32±1.788
F7	2.5:10:2.5/25:100:25	84.45±3.6	13.09±3.1	14.72±1.45	4.74±2.131
F8	3.5:10:2.5/35:100:25	81.53±2.1	13.44±2.2	14.22±2.1	5.65±2.402
F9	2.0:12:2.5/20:120:25	84.13±3.9	15.65±1.9	21.8±1.5	4.56±1.862
F10	2.0:15:2.5/20:150:25	82.41±4.3	16.12±2.9	20.42±1.4	4.84±1.739

(D: PC: CH—drug: phosphatidylcholine: cholesterol ratio, PLLS---Percentage of lipid in liposomal suspension, PCLS----Percentage of cholesterol in liposomal suspension, PDLS---Percentage of drug in liposomal suspension) *Values indicates mean ± S.D. (n=3), **Values indicates mean ± S.D. (n=30).

The particle size and zeta potential of SUVs of both neutral and positive charged liposomes are shown in Table 3. Stearyl amine was found to impart positive charge to the liposomes. The percent drug entrapment of SUVs is shown in Table 3. The PDE was found to be 95%. FTIR report confers no interaction between lipid and the drug (data not shown). *In vitro* release studies indicated that both the formulations (neutral and positive) sustain the drug release up to 10 days (Figure 2). The percent cumulative drug release was observed 77.79 and 67.88 for neutral and positive charged liposomes, respectivelyPositive liposomes show slower release of drug when compared to neutral liposomes. Different kinetic release patterns were evaluated. The log percent cumulative drug released was plotted as a function of log time and the slope of the curves was determined as the values of diffusional release exponent (η). The values of diffusional release exponent (η) from the straight lines were noted to be 0.280 and 0.407 for neutral and positive charged liposomes respectively, which indicated that the release of drug from formulations followed a Fickian pattern (i.e. diffusion-controlled) [17]. Positive charged liposomes demonstrated more effect than the neutral liposomes. This could be because of increase uptake of positive charged liposomes into the liver and further there could be a sustained release of the drug into the plasma. Histopathological data (Figure 5) also provided same information. In the livers treated with CCl_4, there was a significant necrosis of the liver cells including the parenchyma, whereas in all other groups the histology revealed a normal liver. However, the percentage reversal with the *i.v.* solution administration both for SGOT and SGPT although statistically significant, was not reduced to reach the normal levels. However, histological studies revealed normal liver with both neutral and positive charged liposomal formulation

Table 2: Effect of Surface Area on Percent Drug Entrapped

F.C	No.gb (surface area)	D:PC:CH Ratio	PDE*	SIZE(µm)**
F11	10(41.13)	2.0:12:2.5	35.12±3.14	2.12±1.25
F12	30(123.4)	2.0:12:2.5	55.55±4.51	1.54±1.68
F13	50(205.7)	2.0:12:2.5	61.48±4.65	1.95±1.54
F14	80(329.1)	2.0:12:2.5	54.23±3.98	1.61±1.87

(No.gb—number of glass beads taken, D: PC: CH—drug: phosphatidylcholine: cholesterol ratio, PLLS---Percent of lipid in liposomal suspension, PCLS----Percent of cholesterol in liposomal suspension, PDLS---Percent of drug in Liposomal suspension, PDE---Percent drug entrament). *Values indicates mean ± S.D. (n=3), **Values indicates mean ± S.D. (n=30).

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Table 3: Particle size and Zeta potential of SUVs

Formulation	Size	Polydispersity index	Zeta potential	PDE
Neutral SUVs	327.0±15.72	0.221±0.075	-36.9±3.52	>95
Positive SUVs	328.1±18.35	0.273±0.082	+2.16±2.92	>95

(Values indicates mean ± S.D. (n=3))

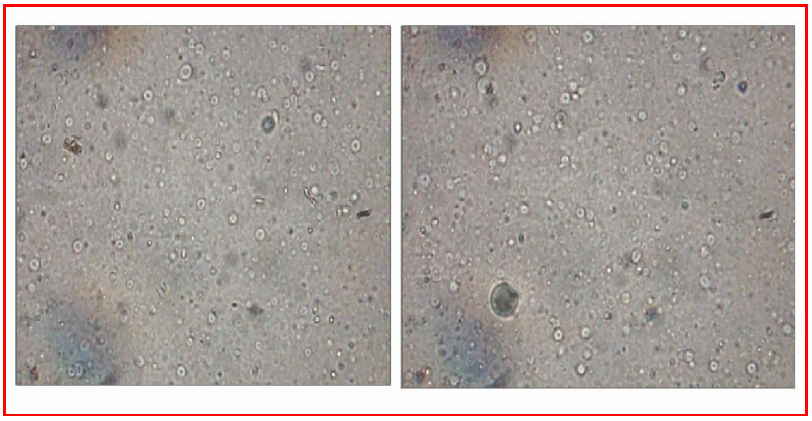

Figure 1: photographs of multilamellar vesicles.

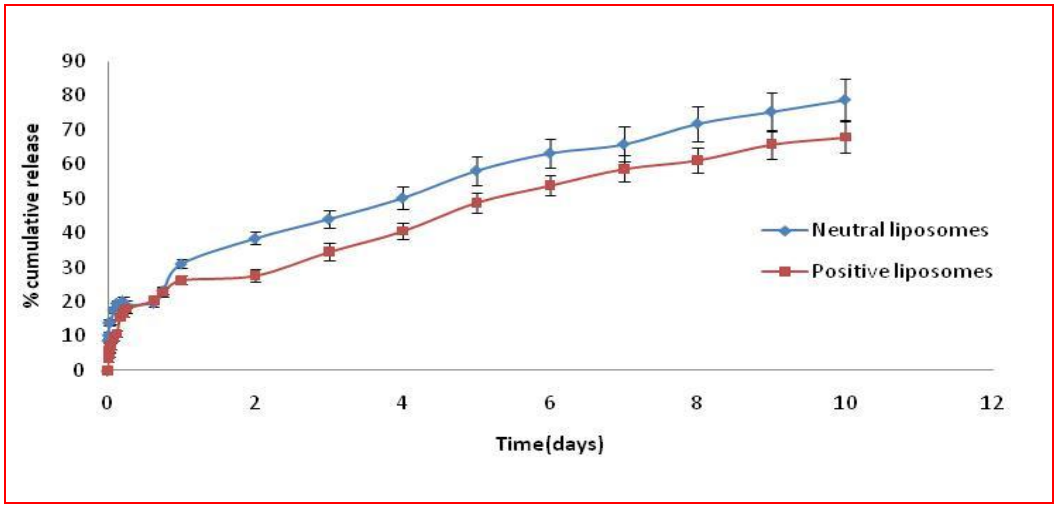

Figure 2: *In Vitro* release of drug from liposomes**,** Mean S.D (n = 3).

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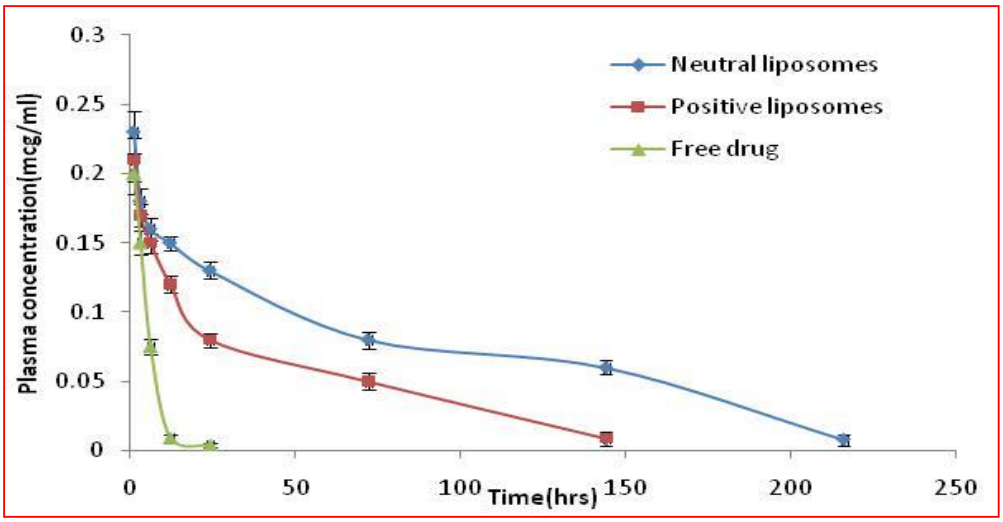

Figure 3: Plasma concentration-time curve of BDMCA after *i.v.* bolus injection of drug loaded liposomes (neutral and positive) and free drug

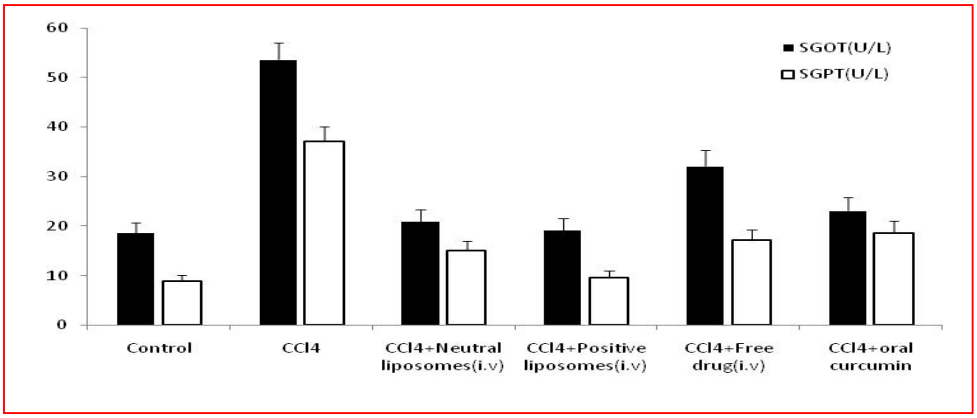

Figure 4: Hepatoprotective activity of the prepared formulations.(Mean±SD (n=3))

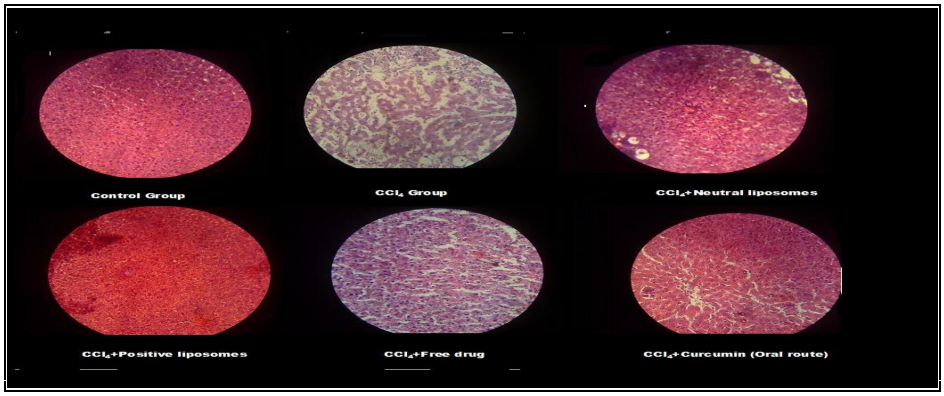

Figure 5: Photographs histopathology slides.

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CONCLUSION

The liposomal formulations were successfully prepared using thin film hydration-sonication method. Neutral and positive charged liposomes sustain the drug release both *in vivo* and *in vitro.* Data conveniently suggests us that the liposomal formulations are more effective than free drug *iv* formulation in achieving the reversal of changes found in the liver upon administration of CCl_4. The enhanced activity of the liposomal formulations could be because of higher uptake of drug into the kupffer cells and sinusoidal endothelial cells, the cells of interest in this study.

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