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Original articl

Improved *in vitro* and *in vivo* hepatoprotective effects of liposomal silymarin in alcohol-induced hepatotoxicity in Wistar rats

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ABSTRACT

Background: Silymarin, a known hepatoprotectant, owing to its poor oral bioavailability, has limited pharmacological effects. The present study was designed to improve its *in vitro* and *in vivo* hepatoprotection and increase its oral bioavailability against alcohol intoxication by formulating it in four different liposomal formulations namely conventional, dicetyl phosphate, stearyl amine and PEGylated liposomes.

Method: The liposomes were prepared using phosphatidylcholine, cholesterol, and silymarin in addition to dicetyl phosphate, stearyl amine and DSPE mPEG 2000 by film hydration method with 5% sucrose as a cryo-protectant. The optimized formulations were studied for their release profile at pH 1.2 and 6.8. Liposomes were studied for *in vitro* protection on Chang liver cells and efficacious liposomes were selected for *in vivo* hepatoprotection study. Further, conventional liposomes were studied for bioavailability in alcohol intoxicated Wistar rats.

Results: The conventional liposomes increased *in vitro* release profile at pH 1.2 and 6.8 and also showed better *in vitro* protection compared to silymarin alone. Conventional and PEGylated liposomes showed better improvement in liver function, better efficacy in combating inflammatory conditions, better improvement in antioxidant levels and reversal of histological changes compared to silymarin alone. Conventional also showed an almost fourfold increase in area under the curve compared to silymarin suspension.

Conclusion: Conventional and PEGylated liposomes of silymarin were found to be more efficacious as hepatoprotective against alcohol-induced hepatotoxicity by its free radical scavenging and anti-inflammatory effects. Conventional liposomes showed enhanced bioavailability compared to silymarin alone.

Introduction

Silymarin is a well-known hepatoprotective agent, widely used in the treatment of various types of liver diseases. However, its poor oral bioavailability [1], poor solubility and unstable nature in the gastric juice [2], limits its pharmacological action. The present study is aimed at studying a phyto-liposomal approach of silymarin against alcohol-induced hepatotoxicity. The liposomal and phytosomal formulation has been in use since decades and provides targeting or delivery of drugs to specific sites in the body. The liposomal approach includes incorporation of drug in the vesicles of lipids without any chemical interaction, whereas phytosomal approach makes the complex of drug-lipid with a chemical interaction. The activity of liposomes in carrying drugs depends upon various factors like composition, rigidity, charge on the membrane, entrapment efficiency, rate of release, stability and distribution inside the body after administration [3,4]. In fact, modifications in the liposomal vesicle structure exhibit superior pharmacological properties when compared to the conventional liposomes, such as hybrid liposome of silymarin

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prepared by film deposition method showed increased oral bioavailability in Beagle dog [5]. Both liposomal and phytosomal formulations have their own specific advantages. The phytosomal approach has been successful in providing stability to silybin (an active constituent of silymarin) in the gastrointestinal environment and liposomal systems have shown selective targetability to inflammatory cells. In the earlier study, the combined approach has shown better efficacy in combating paracetamol-induced hepatotoxicity [6].

The present study was designed to study the interaction of various liposomal vesicle modifying agent namely charge imparting agent Dicetyl phosphate and stearyl amine and stealth (PEGylated) liposomal approach in the phytoliposomes. Further, the characterized formulation was studied for *in vitro* and *in vivo* hepatoprotection.

Materials and methods

Chemicals

Silybin, silymarin, L-α-Phosphatidylcholine (PC, from soybean), minimum essential medium (MEM), fetal bovine serum (FBS) and 3-(4, 5 dimethyl thiazole-2 yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin and triton-X 100 were purchased from Himedia lab Pvt. Ltd. (Mumbai, India). All other chemicals used in the study were of analytical grade.

Cell lines and culture media

Chang liver cells and Human Hepatocellular carcinoma (HepG2 cells) were procured from National Centre for Cell Sciences, Pune, India. The cells were grown in complete MEM and EMEM respectively for Chang and HepG2 cells supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution. Cells were maintained in T25 flasks at 37 °C in a humidified incubator containing 5% CO_2.

Animals

Male Wistar rats of 4–6 weeks age were acclimatized for one week before the study in the experimental room at controlled temperature (23 ± 2 °C) and humidity (50 ± 5%RH) conditions and 12:12 h light and dark cycle. The rats were fed with standard food pellets and water *ad libitum*. The study was conducted after obtaining clearance from the Institutional Animal Ethics Committee of KMC (IAEC NO.- IAEC/KMC/51/2009-2010).

Preparation of liposomes

Development of liposomal formulation of silymarin

The liposomal formulation was prepared as per the method described in the earlier report [6]. Briefly, silymarin (S, 10 mg), phosphatidylcholine (SPC, 180 mg) and cholesterol (C, 15 mg) were dissolved in a methanol-chloroform mixture (1:9) in a round bottom flask [6,7]. The solvent was evaporated in a rotary evaporator at 40 °C under vacuum and a thin film was obtained. The film was dried in a vacuum in desiccator overnight. The film was hydrated with phosphate buffer saline (pH 7.4) containing sucrose 5%w/v as cryoprotectant at 50 °C. The size of the formulation was made uniform by high-pressure homogenization at 20,000 psi. The obtained suspension was frozen overnight at −80 °C, lyophilized and stored at 2–8 °C in airtight containers. For the preparation of other liposomes dicetyl phosphate, stearyl amine and MPEG-DSPE 2000 were added in various ratio with lipid as mentioned in Table 1.

Table 1
Optimization of additives in SPC: Cholesterol: additives with a fixed Sylimarin 10 mg ratio without freeze drying.

Formulation	Molar ratio (SPC:C:P)	%Entrapment
Conventional	6:1:0	57.21
DP1	6:1:0.5	25.04
DP2	6:1:1	47.46
DP3	6:1:1.5	62.7
PEG1	6:1:0.15	17.09
PEG2	6:1:0.3	37.49
PEG3	6:1:0.3	47.49
SA1	6:1:0.5	40.2

Physicochemical characteristics of liposomes

Particle size and zeta potential. The freeze-dried liposomes were re-dispersed in milli-Q water to determine average particle size and polydispersity index (PDI) and zeta potential using Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK).

Estimation of silymarin, drug loading and drug entrapment efficiency in liposomes. C-18 reverse phase column was used in the study. It was procured from Merck (Darmstadt, Germany) with column features: Column-25 cm long, 4 mm inner diameter. The chromatographic conditions used for estimation of silymarin was methanol: water (50:50, pH 3.5, mobile phase), flow rate-1.0 ml/min, detection wavelength-286 nm, detection limit of silymarin-1 μg/ml. A sample of silymarin showed 44.9% of silybin A and 57.31% of silybin B. For analysis of silymarin, the average amount of silybin A and silybin B was taken into consideration. Silymarin in the formulation was quantified by dispersing 2 mg of lyophilized formulation in 2 ml of deionized water. The suspension was centrifuged at 1000 rpm for removing macroparticles. The obtained supernatant was further centrifuged at 64,000 × *g* at 4 °C for 30 min (Beckman Coulter, CA, USA). The supernatant was separated and used for the estimation of free drug content. The resulting pellet was dissolved in 100 μl of 10% Triton X and volume was made 1 ml by mobile phase. The concentration of silymarin was analyzed using high-performance liquid chromatography (HPLC). Percentage entrapment efficiency was calculated using the formula "[Drug present in pellet/drug present in (supernatant + pellet)] × 100". Percentage drug loading of the optimized lyophilized formulation was calculated using the formula "Drug present in formulation/weight of formulation".

Morphology. The liposomes were suspended in deionized water (5 mg/ml) and visualized by transmission electron microscopy using TECNAI 200 Kv TEM (Fei, Electron Optics) at the sophisticated analytical instrument facility for the electron microscope, AIIMS, Delhi.

In vitro drug release (dissolution study). The dissolution study was performed to evaluate the release rate of silymarin from the formulation in acidic pH using HCl (pH 1.2) and phosphate buffer saline (pH 6.8) as per the guideline of US FDA of dissolution study for immediate release solid oral dosage formulation [8]. The dissolution flasks were immersed in a water bath at 37 °C. The medium was stirred at 100 rounds/min. 77 mg of silymarin and the equivalent amount of silymarin in liposomes were added to the surface of the stirred dissolution medium [6]. At 1.5, 3, 4.5, 6, 12 and 24 h time interval, 10 ml samples were collected from the dissolution medium and the same was replaced with fresh medium. The collected medium was filtered using a 0.22 μm syringe filter. 40 μl of the filtrate was injected into HPLC and silymarin amount was measured using standard plat at a wavelength of 286 nm.

In vitro *protection study*

Silymarin and its liposomes were screened for *in vitro* cytotoxicity on HepG2 and *in vitro* protection on Chang liver cells using MTT cytotoxicity assay [6,9]. HepG2 cells were seeded for 24 h. Concentrations of silymarin liposomes were made by dissolving in maintenance media. HepG2 cell lines were pretreated with silymarin and its liposomes for 48 h and percentage viability was evaluated by MTT assay.

The doses of treatments (silymarin and its formulation) were selected based on the earlier reports on Chang Liver cells [6]. The maximum selected concentration of silymarin (62.5 μg) was less than half of its cytotoxicity concentration at which 50% of cells died (CTC50 of silymarin-151.2 μg/ml). The concentration of alcohol (toxicant) was more than CTC50 (7.25%). Chang liver cells were pretreated with silymarin and its liposomes for 24 h. After 48 h, 100 μl of 7.25% of alcohol in media was added to each well except control. The plates were then incubated at 37 °C for 24 h in a CO_2 atmosphere. The media was removed and 100 μl of 1 mg/ml solution of MTT was added for 4th h and absorbance measured at 540 nm. The percentage viability was proportional to the blue colored product (formazan) formed from MTT by succinate dehydrogenase activity of live cells.

Nuclear staining of treated Chang liver cells

Nuclear staining assay of silymarin and its formulations were performed in 24-well plates after plating 2000 cells for 24 h. Treatments were added at 7.5 μg/ml equivalent of silymarin and incubated for 24 h. The media was removed and the plate was washed with phosphate buffer saline (PBS, pH 7.4). After 48 h, 100 μl of 7.25% of alcohol in media was added to each well except control well and incubated for 24 h. Further, ice-cold methanol was added as a fixative and incubated for 20 min. After washing with PBS, Hoechst 33,258 stain (50 μl, 2 μg/ml) was added to each well and incubated at 37 °C for 20 min. The plate was washed thrice with PBS and cells observed under a fluorescent microscope for morphonuclear changes [10]

In vivo *study*

Acute toxicity study of the conventional and PEGylated formulation of silymarin

Acute toxicity was conducted as per OECD guidelines for the testing of chemicals/section 4: Health effects Test No. 425: acute oral toxicity: up and down procedure. Dose selection for toxicity study was based on Acute Oral Toxicity (Guideline 425) Statistical program version 1.0. Throughout 14 days of observation period after drug administration, toxicity parameters related to the neurological profile and any event of mortality was monitored and recorded.

In vivo *hepatoprotection of optimized formulation in alcohol-induced hepatotoxicity model in Wistar rats*

Wistar rats weighing 150–180 g were divided into six animals in five groups [n = 6] *viz.*, sham control (0.25% w/v CMC, 10 ml/kg), alcohol control, silymarin (50 mg/kg), conventional liposome (50 mg/kg equivalent to silymarin) and PEGylated liposome (50 mg/kg equivalent to silymarin).The animals were administered 15 ml/kg of ethanol (45% v/v) orally two times a day for 30 days. Silymarin and its liposomal formulations were administered from day 31 to 45 at a dose of silymarin equivalent to 50 mg/kg *po* in CMC (0.25% w/v) vehicle. A change was made in alcohol dosing, instead of twice a day, alcohol was administered in drinking water at a concentration of 25% v/v. On the 45th day, blood was withdrawn and serum was separated from the blood by centrifuging it at 5000 RPM for 5 min.

Determination of antioxidant status

Assay for glutathione. The glutathione assay was performed by comparing the color formation due to the formation of the derivative by 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB). An equal volume of 5% trichloroacetic acid and the tissue homogenate was centrifuged to get a clear supernatant. The following solutions were incubated for 10 min at room temperature 500 μL of the supernatant, 3 ml of PBS and 500 μL of DTNB. Amount of reduced glutathione was calculated from the absorbance at 412 nm and extrapolated on a standard plot of glutathione. The amount is expressed in μM per mg of protein [11].

Assay of lipid peroxidation. A mixture of 0.5 ml of tissue homogenate 2.5 ml of thiobarbituric acid (TBA), trichloroacetic acid (TCA) and butylated hydroxyl toluene (BHT) was heated at 90 °C for 10 min. The mixture was centrifuged at 2000 rpm for 5 min and absorbance was measured at 525 nm. Lipid peroxidation was expressed as nM of malondialdehyde formed per mg of tissue [12].

Assay for super oxide dismutase (SOD) [13]. Tissue homogenate (50 μL) was added to a mixture of 1850 μl of carbonate buffer, and 100 μl of adrenaline in a cuvette and the change in absorbance was recorded at 480 nm (A_0–A_{60}). Change in absorbance was extrapolated using the standard plot to find out the SOD activity, which was expressed in units/mg of protein.

Assay of total thiols [11]. A mixture of 100 μl of tissue homogenate, 100 μl of tris EDTA solution, 40 μl of DTNB and 3.16 ml of methanol was centrifuged at 2000 rpm. The absorbance of the supernatant was taken at 412 nm. Amount of total thiols was expressed in nM/mg of protein.

Bioavailability study in alcoholic Wistar rats

Bioavailability study of silymarin (equivalent to silybin) was performed by HPLC using a chromatographic condition as described above in Section "Physicochemical characteristics of liposomes". The study was performed in alcohol intoxicated Wistar rats. The hepatotoxicity in rats was induced by alcohol as mentioned in Section 2.5.2. Twelve Wistar rats were grouped in two and fasted for 8 h. Silymarin was administered orally in 0.25% carboxymethylcellulose (CMC) suspension and conventional liposomes in distilled water. The dose for silymarin and its formulation was 200 mg/kg equivalent of silybin. The time points for blood withdrawal (250 μl) were 15 min, 30 min, 1 h and 2 h. Internal standard used in the study was α-naphthol, which was added in a quantity of 5 μl for 100 μl of separated plasma. Extraction of the drug from plasma was performed by addition of 300 μl chilled methanol:acetonitrile (50:50). The protein was precipitated and the mixture was centrifuged at 14,000 rpm for 10 min. To increase the sensitivity, injection volume for the supernatant was 40 μl. The standard plot was made in a similar condition. The peak concentration, peak time, AUC was calculated by non-compartment modeling using WinNonline software, Pharsight Corporation, CA, USA.

Statistical analysis

Results were analyzed by one way ANOVA followed by Tukey's *post hoc* method of analysis except for "*In vitro* protection study against alcohol-induced toxicity" where two way ANOVA followed by Tukey's *post hoc* method of analysis was used. The statistical analyses was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, California, USA (Evaluation version).

Results

Preparation of optimized silymarin liposomes

Preformulation studies

Physical mixtures (silymarin and excipients) did not show any change in DSC and drug content of silymarin. This is indicative of the drug's physical and chemical stability with the excipients. Excipients used in the formulations were biocompatible and biodegradable with high purity grade.

Preparation of liposomes

Molar ratio of SPC:C:P selected for optimization were 6:1:0, 6:1:1.5, 6:1:0.3 and 6:1:0.5 respectively for conventional, DP, PEG and SA liposomes of silymarin, where percentage entrapment was found to be 57.21%, 62.7%, 47.49% and 40.2% respectively. SA formulation showed agglomeration of particles, so the addition of a higher amount of SA was not considered. The optimized formulation after lyophilization showed a change in percentage entrapment to 62.29%, 44.72%, 50.50% and 58.94% conventional, DP, PEG and SA liposomes of silymarin respectively (Table 2).

Characterization of liposomes

DSC thermogram showed a shift of endothermic peak of around 40–50 °C in liposomes and silymarin. The endothermic peak of silymarin was obtained at 257.38 °C. The endothermic peaks liposomes conventional, DP, SA and PEG liposomes thermogram were obtained at 207.66 °C, 205.30, 214.26 and 213.74 °C respectively. Transmission electron microscopy (TEM) for surface morphology was performed for lyophilized liposomes after slightly shaking in water. Particle appeared to be spherical and uniform in shape (Fig. 1).

Dissolution in HCl (pH 1.2) and phosphate buffer saline (pH 6.8)

At pH 1.2, only conventional liposomes showed almost complete release (99.3 ± 1.8%) after 24 h. The percentage cumulative drug release by remaining liposomes *viz.*, DP, PEGylated, SA were found to be 79.3 ± 4.5, 57.6 ± 0.8, 31.4 ± 0.6 respectively. The minimum release was observed in free drug (silymarin alone), which was 11.3 ± 1.3 after 24 h. At pH 6.8, the percentage cumulative drug release from the liposomes *viz.*, Conventional, DP, PEGylated, SA were found to be 95.5 ± 1.7, 29.7 ± 1.9, 98.7 ± 0.4, 33.8 ± 1.61 respectively while silymarin alone was 17.8 ± 0.5% after 24 h (Fig. 2).

In vitro *cytotoxicity on HepG2 cell line*

Treatment with formulations namely Conventional, PEG and DP showed significant ($p < 0.05$ decrease in percentage viability at 15.625, 31.25 and 62.5 μg/ml compared to silymarin alone (Table 3).

Table 2
Characteristics of final formulations after freeze drying.

Liposomes	Particle Size	PDI	Zeta potential	Entrapment efficiency	Effective entrapment Ratio (SPC:C:P)	Drug loading
Conventional	322 nm	0.563	−70.5 mv	58.94%	6:1:0	11.59%
Dicetyl Phosphate	756 nm	0.607	−77.3 mv	62.29%	6:1:1.5	0.5%
MPEG-DSPE2000	146.9 nm	0.958	−47.4 mv	50.50%	6:1:0.45	2.3%
Stearyl amine	1377 nm	0.767	−64.7 mv	44.72%	6:1:0.5	0.14%

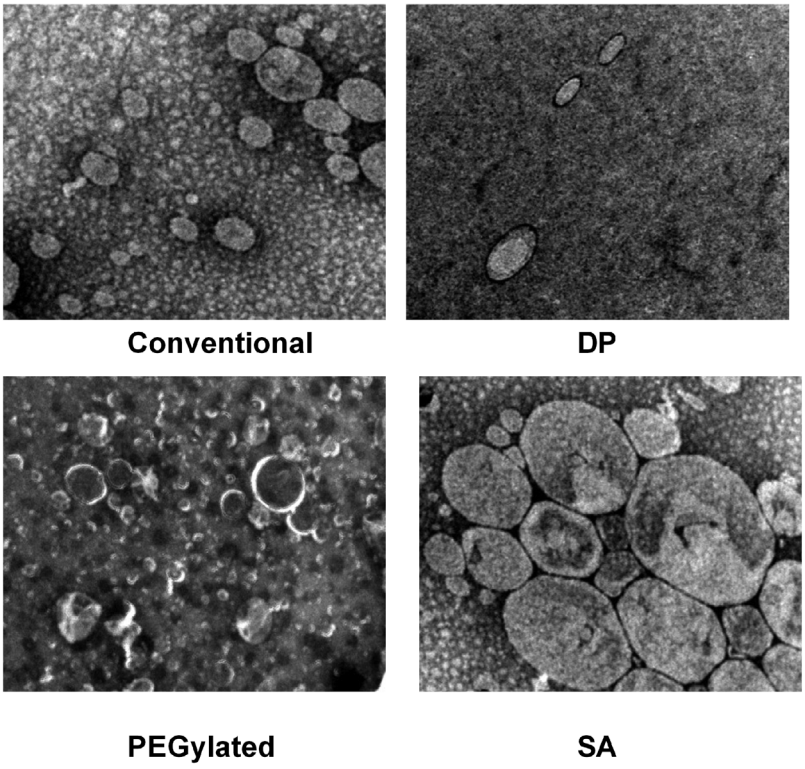

Fig. 1. TEM of liposomes. TEM images of Conventional liposomes, DP (dicetyl phosphate) liposomes, PEGylated liposomes and SA (stearyl amine) liposomes.

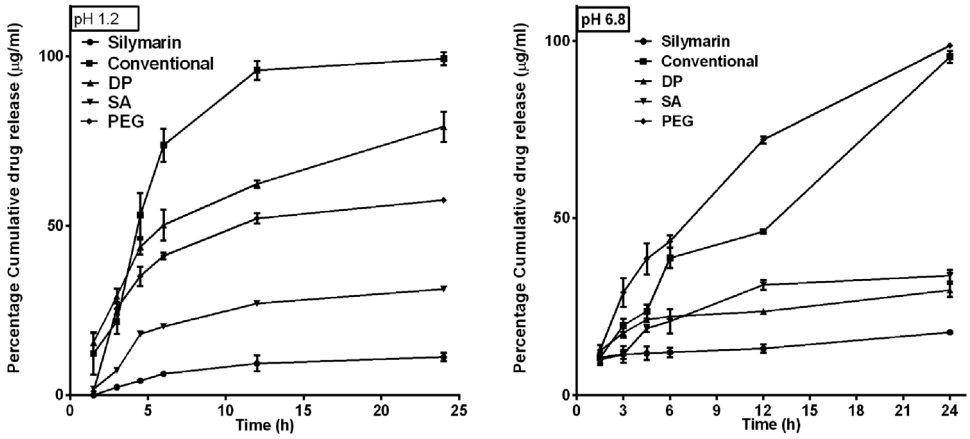

Fig. 2. Release study of silymarin and its liposomes at pH 1.2 and 6.8. All the values are mean ± SEM of six readings of percentage cumulative release of silymarin, conventional liposomes, DP (dicetyl phosphate) liposomes, SA (stearyl amine) liposomes and PEGylated liposomes at 1.5, 3, 4.5, 6, 12 and 24 h. Treatment alone showed more than 60% viability at the highest tested concentration.

Table 3
Percentage viability of silymarin and its formulation on HepG2 cells.

Concentration (µg/ml of silymarin)	Silymarin	Conventional	PEG	DP	SA
7.8125	105.12 ± 7.21	89.00 ± 3.01	96.44 ± 7.22	95.88 + 7.90	102.22 ± 9.80
15.625	115.04 ± 8.82	79.01 ± 5.10[a]	86.40 ± 9.67[a]	85.74 ± 9.80[a]	99.88 ± 3.80[b]
31.25	102.41 ± 0.52	70.51 ± 2.36[a]	70.24 ± 1.00[a,b]	76.02 ± 3.15[a]	98.88 ± 7.57[b,c,d]
62.5	101.98 ± 9.82	65.70 ± 5.34[a]	41.22 ± 4.19[a]	60.31 ± 5.51[a,c]	83.98 ± 4.90[c,d]
125	101.48 ± 0.52	53.43 ± 1.71[a]	37.28 ± 1.45[a]	27.96 ± 3.86[a,b,]	74.34 ± 9.80[a,b,c,d]
250	52.91 ± 3.66	34.59 ± 0.52	35.34 ± 0.53	34.59 ± 0.76	45.25 ± 4.02[x]
500	35.72 ± 0.35	34.10 ± 0.15	33.55 ± 0.34	33.34 ± 0.43	35.31 ± 1.15
1000	34.93 ± 0.52	32.72 ± 0.55	33.45 ± 0.21	33.76 ± 1.37	32.31 ± 0.58

All the values are mean ± SEM of three trials in triplicate. Data are analysed by two way ANOVA followed by Tukey's test. In row wise: [a]$p < 0.05$ compared to silymarin, [b]$p < 0.05$ compared to Conventional, [c]$p < 0.05$ compared to PEG, [d]$p < 0.05$ compared to DP. F (DFn, DFd):: for Interaction: F (28, 80) = 5.467, For Concentration: F (7, 80) = 151.5, For Treatment: F (4, 80) = 49.07.

In vitro *study on Chang liver cell line*

In vitro *protection study against alcohol-induced toxicity*

Treatment alone at the highest concentration (62.5 µg/ml) showed percentage viability 100.75 ± 9.05, 78.31 ± 6.34, 94.11 ± 9.23, 85.08 ± 5.66, 75.05 ± 4.36 and 64.07 ± 3.06 respectively for silymarin, PEG, DP, SA, conventional and equivalent concentration of lecithin.

When cells were challenged with 7.25% alcohol, the percentage viability observed was 31.2% compared to control cells, which had 100% viability. Silymarin showed maximum protection at 7.8 µg/ml (percentage viability 60.53%). As concentration increased, the protection was lesser. The same trend was observed for all formulations DP, SA, and PEG. As the concentration increased, the toxicity also increased. This might be due to an increase in the amount of the excipient or due to increased cytotoxicity of silymarin itself. The significant protection was observed by the tested formulations at different concentrations. Compared to silymarin, PEG and DP formulation showed significant ($p < 0.05$) protection at 7.8 µg/ml, SA and Conventional at 15.60, 31.25 and 62.50 µg/ml while equivalent concentrations of lecithin showed significant ($p < 0.05$) decrease in protection at 15.6 µg/ml (Fig. 3).

Nuclear staining study

Nuclear morphological changes were found to be remarkable in toxicity. Control cells had an intact round or oval nucleus without condensation. The cytoplasm of the control cells was normal with the intact cell membrane and without any cytoplasmic disintegration. Toxicant treatment showed condensation in the nucleus and

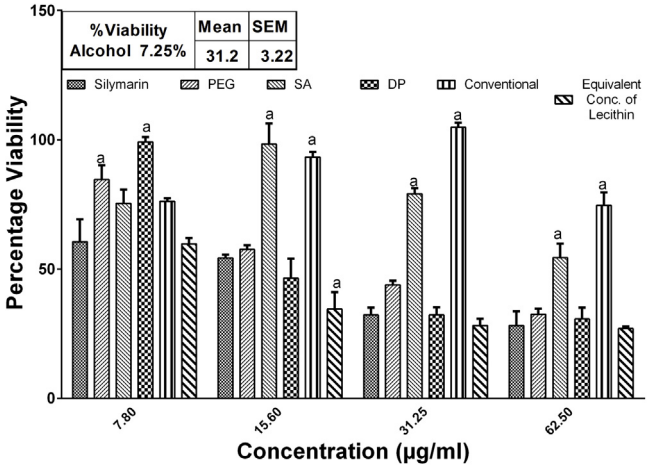

Fig. 3. Protective effect of developed formulation on alcohol-induced toxicity in Chang liver cells by MTT assay. Graph shows percentage viability of silymarin and its equivalent concentration in various liposomes namely PEG (PEGylated), SA (stearyl amine), DP (dicetyl phosphate), conventional liposomes and an equivalent concentration of lecithin (L-α-Phosphatidylcholine). All the values are mean ± SEM of three trials in triplicate. Data is analyzed by two-way ANOVA followed by Tukey's multiple comparison test, where the column represent treatment and rows represent concentrations of treatment. The significance values are [a]$p < 0.05$ compared to silymarin, F (DFn, DFd):: for interaction-F (15, 48) = 12.83 and $p < 0.0001$; for concentration F (3, 48) = 68.13 and $p < 0.0001$; for treatment F (5, 48) = 77.72 and $p < 0.001$. Adjusted p values compared to silymarin were at $p < 0.0001$ except for PEG at 7.8 µg/ml $p = 0.0042$ and for SA at 61.5 µg/ml $p = 0.0014$.

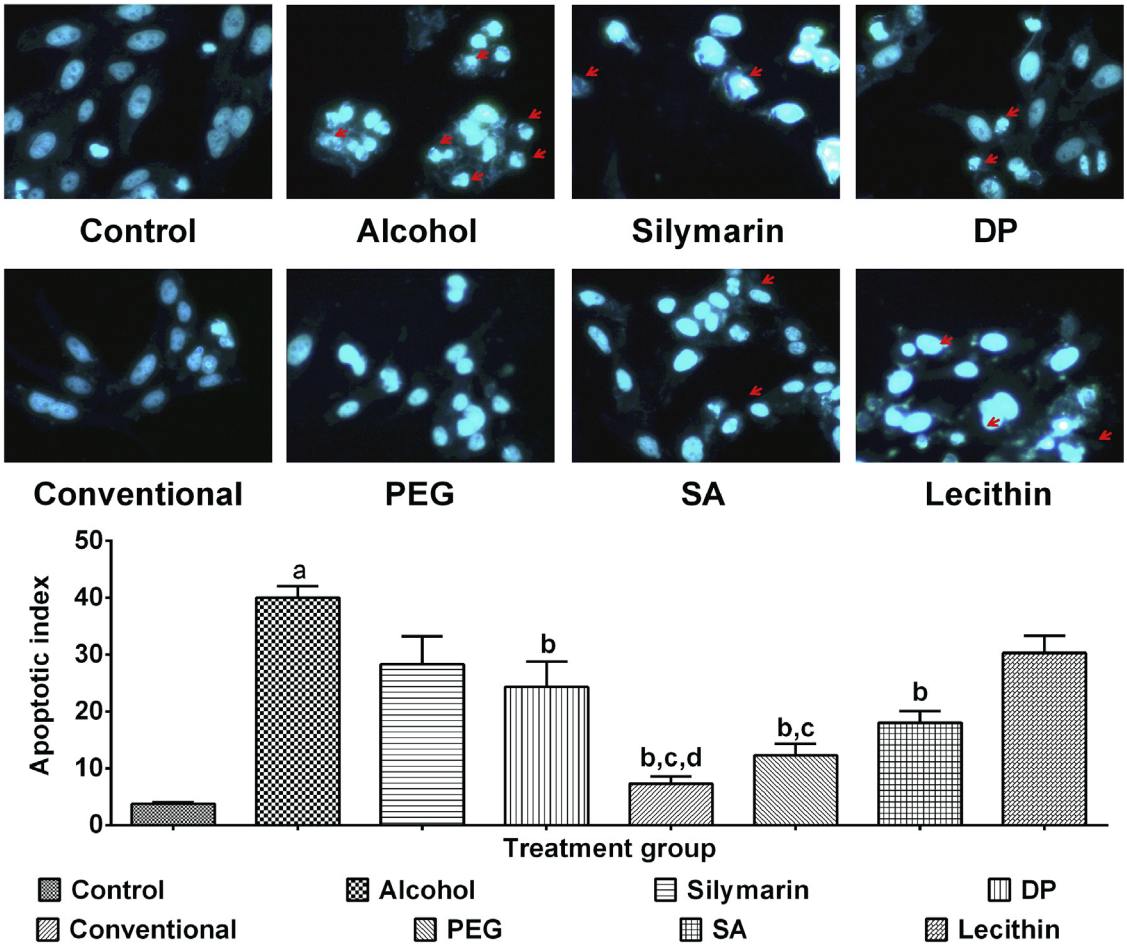

Fig. 4. Hoechst 33342 nuclear staining for the effect of formulations against alcohol-induced toxicity on Chang liver cells. Nuclear staining images of silymarin (7.8 μg/ml) and its equivalent concentration in various liposomes namely PEG (PEGylated), SA (stearyl amine), DP (dicetyl phosphate), conventional liposomes and an equivalent concentration of lecithin as in liposomes (L-α-Phosphatidylcholine). Concentration of Alcohol-7.25%. Magnifications 400×; Arrows indicates morphonuclear changes. All the values are mean ± SEM of three independent trials performed in triplicate. In each trial, 100 cells were observed. Data is analyzed by one-way ANOVA followed by Tukey's multiple comparison test, where $^{a}p < 0.0001$ compared to control, $^{b}p < 0.05$ compared to alcohol (p value:: DP: $p = 0.0249$, conventional and PEG: $p < 0.0001$, SA: $p = 0.0013$), $^{c}p < 0.05$ compared to silymarin (p value for conventional and PEG 0.0020 and 0.0216 respectively), $^{d}p < 0.05$ compared to DP (p value -0.0135). F values: F (DFn, DFd) F (7, 16) = 18.4.

disrupted membrane with cytoplasmic disintegration. Pretreatment of silymarin and its liposomal formulations prevented these morphological changes in the nucleus. Maximum protection was observed with conventional liposomal pretreatment (Fig. 4).

In vivo *study*

Acute toxicity study of conventional and PEGylated liposomes of silymarin

Formulations were safe up to 2000 mg/kg equivalent to silymarin. No toxic signs were observed in mice with both the formulations.

Biochemical estimation in serum

Alcohol administration increased the levels of AST, ALT, total bilirubin significantly ($p < 0.05$) in the alcohol-treated control group. The raised AST level was significantly ($p < 0.05$) lowered by silymarin, and its liposome. ALT level was significantly ($p < 0.05$) lowered by both liposomes *viz.*, conventional and PEGylated. ALP and direct bilirubin levels were not significantly changed. Albumin level decreased significantly in the alcohol control group, which showed that the synthetic function of the liver was compromised. However, treatment with silymarin and its liposome significantly improved the synthetic function of the liver by elevating albumin level (Fig. 5).

Antioxidant parameters in liver tissue homogenate

Chronic administration of alcohol affected the antioxidant defense system of the liver. Catalase level was significantly ($p < 0.05$) lowered due to the chronic administration of alcohol. Both liposomal formulations showed better protection than silymarin alone by elevating catalase level. The same trend was observed in the level of SOD. Total thiols and GSH levels were depleted due to chronic alcohol administration in the control group while silymarin and its liposome treatment restored these levels significantly ($p < 0.05$). Free radical generation and its impact were measured in terms of malondialdehyde (MDA) formation. The MDA level formation was more in the alcoholic control group. This was significantly lowered by silymarin and its liposomal treatments (Fig. 6).

Inflammatory condition in liver tissue

Chronic administration of alcohol resulted in a marked rise in inflammatory markers in the liver homogenate. The IL-6 level in the sham animal was found to be 72.71 pg/mg of protein. Due to chronic administration of alcohol, IL-6 level increased in control animals, more than two-fold to 201.5 pg/mg of protein. Silymarin (122.6 pg/mg of protein) and its liposomes (53.43 for conventional and 72.89 pg/mg of protein for PEGylated liposomes) significantly ($p < 0.05$) reversed the rise in the levels of IL-6 (Fig. 7).

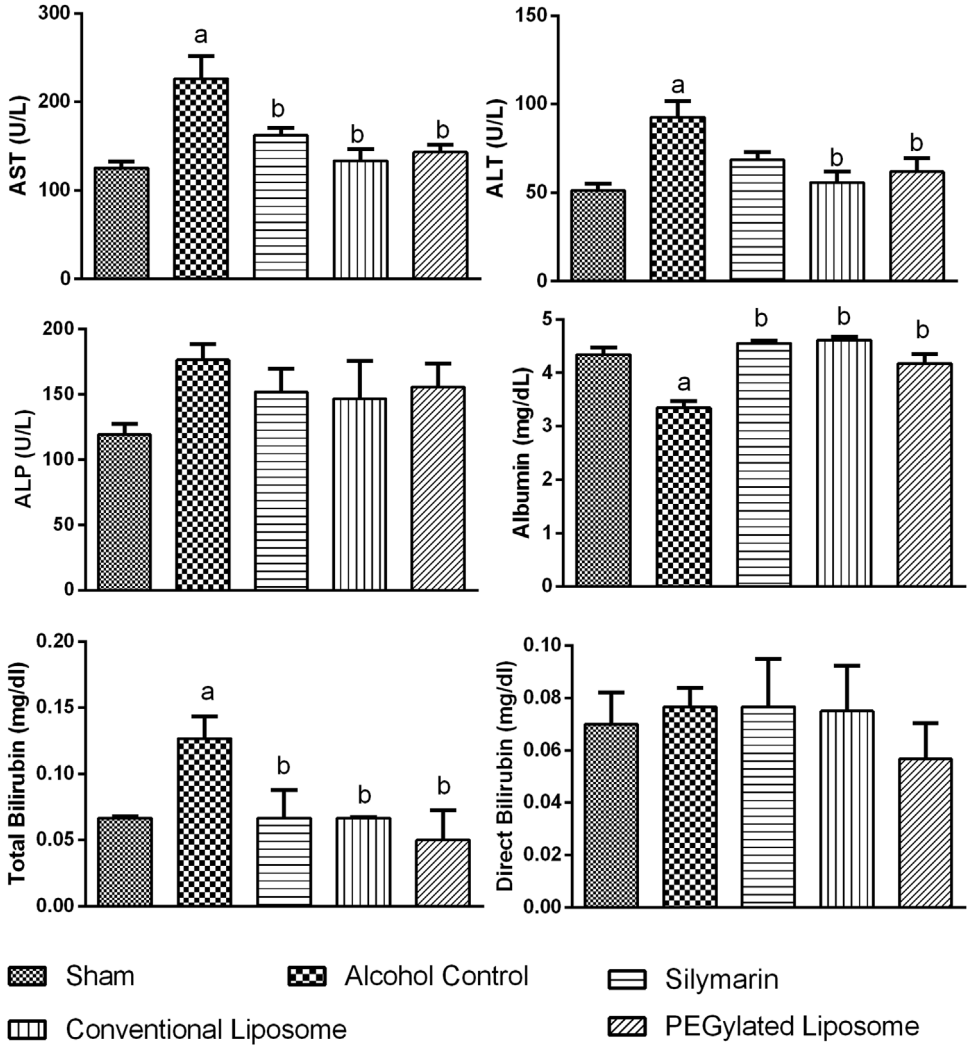

Fig. 5. Effect of silymarin and its liposomes on biochemical parameters of alcohol-induced liver damage. Various parameters of the liver function test were evaluated in serum. All the values are represented as mean ± SEM of six animals, where $^{a}p < 0.05$ compared to sham, $^{b}p < 0.05$ compared to alcohol control, $^{c}p < 0.05$ compared to silymarin.

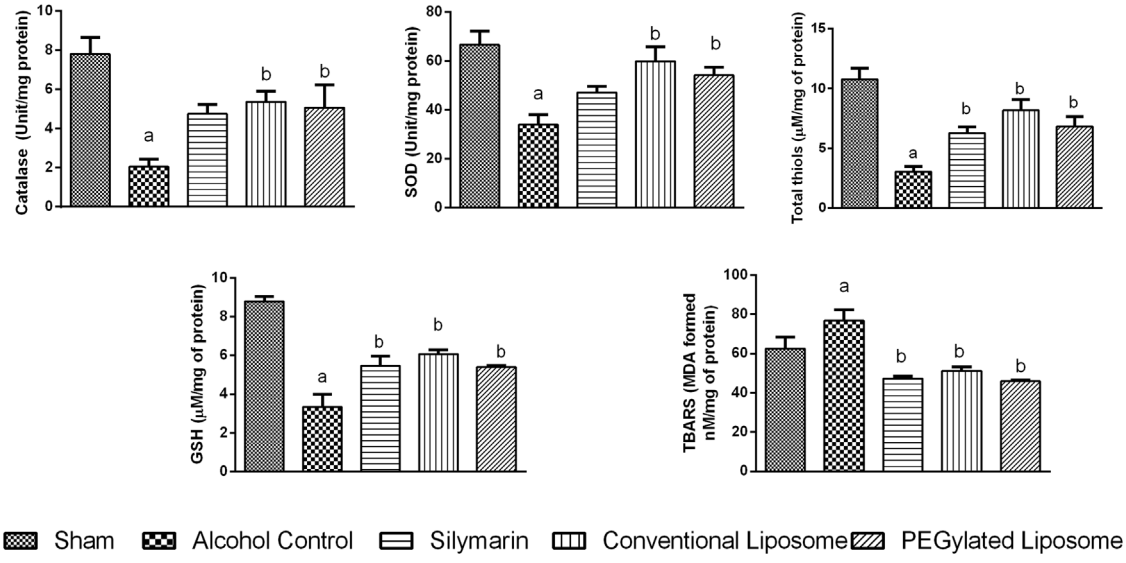

Fig. 6. Protective effect of silymarin and its liposomes on antioxidant status of alcohol-induced hepatotoxicity. Antioxidant parameters were evaluated in the supernatant of liver homogenate. All the values are represented as mean ± SEM of six animals, where $^{a}p < 0.05$ compared to sham, $^{b}p < 0.05$ compared to alcohol control, $^{c}p < 0.05$ compared to silymarin.

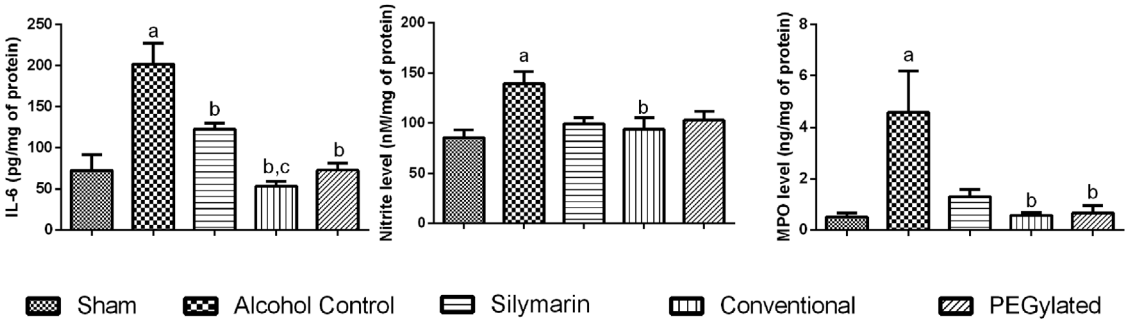

Fig. 7. IL-6, nitrite and MPO levels in liver tissue. All the values are represented as mean ± SEM of six animals, where [a]$p < 0.05$ compared to sham, [b]$p < 0.05$ compared to alcohol control, [c]$p < 0.05$ compared to silymarin.

Chronic administration of alcohol resulted in increased activity of macrophages and neutrophils, which resulted in a series of inflammatory reaction. The rise in nitrite level in tissue is one among them. The level of nitrite in liver homogenate of the alcohol control group (139.4 nM/mg of protein) significantly ($p < 0.05$) increased compared to sham group (85.69 nM/ mg of protein). Silymarin and PEGylated liposome prevented a rise in the level of nitrite, but the values were not significant (99.41 and 102.8 nM/mg of protein respectively). Treatment with conventional liposomes of silymarin (94.02 for conventional) significantly prevented the rise in nitrite level in liver homogenate compared to alcohol control group (Fig. 7).

The significant increase in levels of MPO in the liver homogenate of the alcohol control group (4.57 ng/mg of protein) compared to sham group (0.51 ng/mg of protein) indicated infiltration of neutrophil into the liver. The rise in MPO level was significantly prevented by the pretreatment of silymarin and its liposomes. MPO levels for silymarin conventional and PEGylated group were found to be 1.3, 0.58 and 0.66 ng/mg of protein respectively, while the value for the control group was 4.57 ng/mg of protein (Fig. 7).

Histopathology

Alcohol administration to animals caused moderate damage to the liver. Macroscopic findings revealed enlargement of the liver. Fatty accumulations were observed with congestion in sinusoids and scattered spotty necrosis was noted. The conventional and PEGylated liposomes of silymarin showed better improvement in these parameters compared to silymarin alone (Fig. 8).

Bioavailability study in alcoholic rats

The recovery of silybin A and silybin B (silybin isomers) was found to be 94 to 96%. The retention time was 17.7 and 19.8 min for silybin A and silybin B. The linearity range used for the standard plot of silybin was 30 ng/ml to 10 mg/ml (r^2 0.9999). The concentration of silybin was evaluated by the average of the area under the curve of both isomers. The percentage recovery for internal standard (α-naphthol) was 98%. The C_{max} obtained from the bioavailability study was 100.45 ± 3.45 ng/ml for silymarin, with a t_{max} of 0.5 h. The C_{max} was increased in conventional liposomal formulation administered group to 604.48 ± 14.53 ng/ml without any change in t_{max}. The half-life of silymarin was found to be 1.18 ± 0.10 h, while for

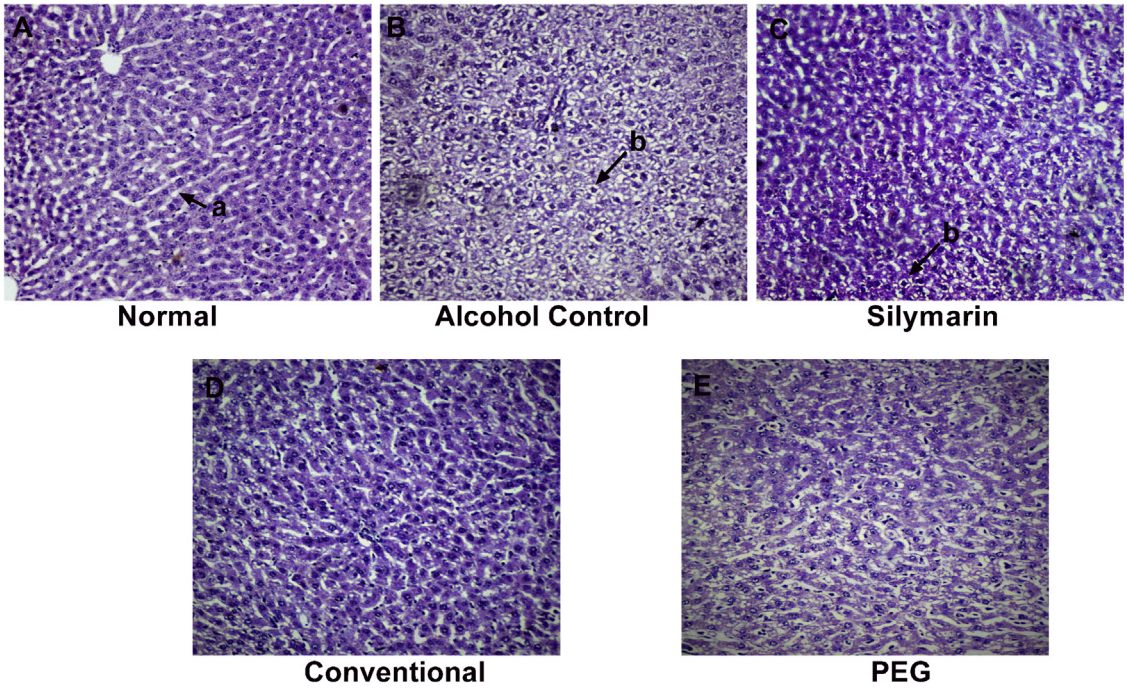

Fig. 8. Histology in alcohol-induced toxicity in Wistar rats. Images represent hematoxylin and eosin images of liver histology at 10X objective. A. Sham, B. Alcohol control, C. Silymarin, D. Conventional, E. PEGylated. Symbol "a" represent sinusoids, "b" fatty accumulation.

Table 4
Pharmacokinetic parameters of silymarin (equivalent to silybin) in alcoholic rats.

Pharmacokinetics parameters	Silymarin Mean ± SEM	Conventional liposomes Mean ± SEM
C_{max} (ng/ml)	100.45 ± 3.45	604.48 ± 14.53
t_{max} (h)	0.50 ± 0.0	0.50 ± 0
AUC_{0-2} (h*ng/ml)	104.64 ± 3.39	430.64 ± 8.52
AUC_{0-inf} (h*ng/ml)	154.51 ± 7.86	464.37 ± 9.3
Elimination rate constant (K_e) (1/h)	0.61 ± 0.05	1.04 ± 0.004
Half-life (h)	1.18 ± 0.10	0.66 ± 0.002
$AUMC_{0-t}$ (h*h*ng/ml)	92.42 ± 2.79	342.22 ± 6.50
$AUMC_{0-inf}$ (h*h*ng/ml)	204.57 ± 11.15	431.64 ± 9.48
Clearance (ml/h)	1450.72 ± 48.58	431.59 ± 8.96
Volume of Distribution (V_d) (ml)	1860.95 ± 99.76	279.00 ± 8.28
Mean Residence $time_{0-t}$ (MRT) (h)	0.88 ± 0.01	0.79 ± 0.001
MRT_{0-inf} (h)	1.47 ± 0.05	0.93 ± 0.01

All the values are mean ± SEM of six animals.

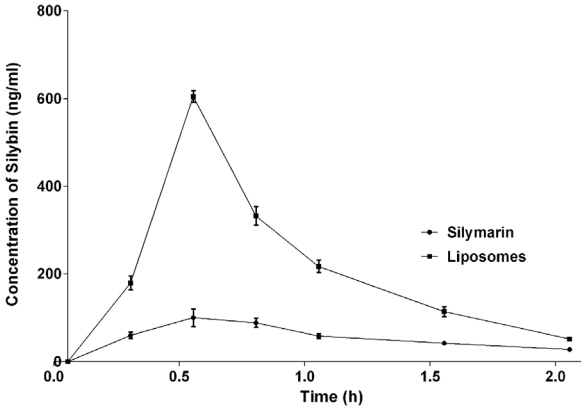

Fig. 9. AUC of silymarin and conventional liposome (equivalent to silybin) in alcoholic rats. All the values are mean ± SEM of six animals.

formulation it decreased to 0.66 ± 0.002 h. The area under the curve (AUC) of the formulation was more than three times higher than silymarin alone (Table 4, Fig. 9).

Discussion

Silymarin is a well-known hepatoprotective agent. It has a poor intestinal absorption, high excretion rate, and hepatobiliary circulation, leading to low bioavailability. Lack of selectivity and low bioavailability from current dosage forms are the major challenges for this drug. Therefore, the phytosomal and liposomal formulation was adopted for silymarin to increase its bioavailability. Since liposomes have high targetability to the immune cells, the developed formulation will be useful in the inflammatory stage of liver damage.

The encapsulation percentage of silymarin in a molar ratio of 6:1 was found to be maximum in the earlier study [6]. Therefore, the same ratio was selected for the development of the charged and PEGylated formulations. These developed formulations were characterized for drug-lipid interaction using DSC spectral data. The spectral data showed the shift in the endothermic peak of silymarin in liposomes, which indicated an interaction between lipid and drug in the liposomal formulation. The produced liposomes were found to be spherical, as seen in the TEM image. The present study confirms the earlier reported findings, where similar interaction between lipids and silymarin in the liposomal formulation was observed [6]. In the present study, the particle size of liposomes was in nanometer range except for SA-liposomes, which was in the micrometer range. Small size liposomes have longer circulation half-life and they are slowly cleared by the reticuloendothelial system. Thus it might increase the chances of more interaction with hepatocyte [14]. The surface charge of the liposomes is an important parameter for physical stability. Normally, the presence of the surface charges of the particles induces electrostatic repulsion among the particles to prevent them from agglomeration. Zeta potential evaluates the surface charge of the nanoparticles. The low zeta potential (in the range between +30 mv to -30 mv) results in physical instability of nano-formulation [15]. In the present study, the zeta potential of the formulation was found to be in a stable range (<−30 mv), which suggested the physical stability of the formulation. The release rates of these liposomes were performed at pH1.2 (made by HCl) and pH 6.8 (made by phosphate buffer saline). The percentage cumulative drug release was significantly high for the conventional liposome in pH conditions of 1.2 and 6.8, although the complete release of silymarin from this formulation happened at pH 6.8 after 24 h. In the acidic pH, we found that the Dicetyl phosphate (DP)-liposomes exhibited a statistically higher cumulative drug release. On the other hand, the PEGylated liposomes showed a higher percentage of cumulative drug release at pH 6.8. However, silymarin (alone) exhibited the lowest solubility in both pH conditions. This finding suggested that the formulation had increased the solubility of silymarin. The present dissolution study results confirm the earlier reports on silymarin and conventional liposomal formulation for their release profile, where the similar trend of release profile is reported at pH 1.2 and 7.4. [6].

Percentage viability of HepG2 cells decreased dose-dependently after the treatment with the liposomal formulations except for DP liposomes. Further, this effect was better than silymarin, which showed lesser efficacy in terms of cytotoxicity. Thus, silymarin in the liposomal form showed increased activity against HepG2 cells.

To examine the protective role of silymarin and its liposomes, Chang liver cells were chosen. There was a significant increase ($p < 0.05$) in the cell viability observed by silymarin liposome pretreated cells as compared to the alcohol challenged cells. The nuclear staining images also confirmed that the liposomes had a clear role in protecting the Chang liver cells from alcohol-induced effects. These *in vitro* protective effects by a liposomal form of silymarin was found to be more than silymarin alone. Similar reports on the protective effect of silymarin and conventional liposomes are available against paracetamol-induced toxicity to Chang cells [6].

The second objective of the study was to understand the effects of silymarin liposomes in protecting against alcohol-induced hepatotoxicity and compared to silymarin alone. Chronic alcohol ingestion produced liver damage [16], marked by raised levels of the LFT parameters *viz.*, AST, ALT, total bilirubin significantly ($p < 0.05$) in the alcohol-control group. Both the liposomal formulations of silymarin were found to be more effective than silymarin in lowering the ALT level. Other parameters namely AST and total bilirubin were also improved by silymarin and its liposome. Albumin level decreased significantly in the alcohol control group, which indicated that the synthetic function of the liver was compromised. Chronic administration of alcohol is known to produce oxidative stress to cell [16]. Hepatocytes are the main site for priming of alcohol-induced oxidative stress, which starts by inducing a hypoxic condition followed by a depletion in GSH and S-adenosine methionine levels [16]. A similar finding has been observed in our study. The depleted GSH, total thiols, catalase and SOD levels were observed in the alcohol-intoxicated group. GSH and total thiols level were significantly ($p < 0.05$) improved by silymarin and its liposomes while catalase and SOD levels were improved by only liposomes and not by silymarin suspension, which indicates that the liposomes are more active. Alcohol intoxication mediated decrease in the antioxidant system is also

accompanied by increased reactive oxygen species (ROS) formation by cytochrome p45 0-2 E1(CYP2E1) of mitochondria leading to the initiation and propagation of lipid peroxidation [16]. Similar results obtained in the present study where alcohol intoxication increased the TBARS level (measured by the levels of malondialdehyde levels). Treatment with silymarin and its liposomes was significantly able to ($p < 0.05$) prevent the rise in MDA level. The product of lipid peroxidation *i.e.*, malondialdehyde, forms an immunogenic adduct with transport protein, which in turn initiates the humoral and cellular immune responses [16]. Chronic administration of alcohol resulted in a marked rise in inflammatory markers in the liver homogenate, which was assessed by monitoring the levels of IL-6, MPO and nitrite levels. Conventional liposomes were found to be most active in preventing the increase in IL-6, MPO and nitrite concentrations in liver tissue. This might be due to the highest drug loading capacity of conventional liposomal formulation compared to other tested formulations. PEGylation approach was selected to make it long circulating and thereby providing more hepatoprotective effects. However, no significant differences were seen in hepatoprotective effects of conventional and PEGylated liposomes. Thus, for the bioavailability study, only conventional liposomes were selected.

The above efficacy study of conventional liposomal silymarin was supported by increased bioavailability of silymarin in alcohol intoxicated Wistar rat. The earlier reported bioanalytical method was used in the present study [6]. Extraction of the drug from the biological matrix is one of the crucial steps in a pharmacokinetic study. Protein precipitation method was implied for extraction of silymarin from plasma, which can be justified by the fact that hydrophilic drugs have better recovery from the biological matrix (like plasma) using protein precipitation method than LLE method [17]. Another crucial step in bioanalytical method development involves the selection of internal standard. α-Napthol was found to be well resolved from the marker compound of silymarin (silybin A and silybin B) and was also used as an internal standard in the previous studies [6,18] for the bioanalytical study of silymarin. A bioavailability study was performed in the alcohol-intoxicated male Wistar rats. Only conventional liposome was selected for bioavailability study based on their hepatoprotective profile. Based on the literature, 200 mg/kg equivalent to silybin was selected as the dose for silymarin and liposomal formulation [19]. The peak plasma concentration of silybin was achieved in 0.5 h in alcoholic rats. In the previous pharmacokinetic report of silymarin in normal rats, t_{max} was found to be the same as reported in this study. However, increased values are seen for C_{max} and AUC [6]. These changes in the parameters could be due to the decrease in gastrointestinal absorption in alcohol-intoxicated rat [20]. In the present study, the C_{max} increased six fold by conventional liposomes of silymarin compared to silymarin alone. This increase in C_{max} was accompanied by a four-fold rise in AUC level and a decrease in half-life. In the earlier study, in normal animals, a similar formulation is reported to increase Cmax by five-fold and AUC by three-fold [6]. The half-life was same as in normal rat which can be justified by the fact that liposomes were cleared selectively by Kupffer cells.

Conclusion

Liposomal formulations of silymarin is a suitable candidate and can be used as a liver protectant. Conventional and PEGylated liposomes of silymarin showed promising hepatoprotective effect among the tested liposomes.

Acknowledgements

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