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Silymarin liposomes improves oral bioavailability of silybin besides targeting hepatocytes, and immune cells

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ABSTRACT

Background: Silymarin, a hepatoprotective agent, has poor oral bioavailability. However, the current dosage form of the drug does not target the liver and inflammatory cells selectively. The aim of the present study was to develop lecithin-based carrier system of silymarin by incorporating phytosomal–liposomal approach to increase its oral bioavailability and to make it target-specific to the liver for enhanced hepatoprotection.

Methods: The formulation was prepared by film hydration method. Release of drug was assessed at pH 1.2 and 7.4. Formulation was assessed for *in vitro* hepatoprotection on Chang liver cells, lipopolysaccharide-induced reactive oxygen species (ROS) production by RAW 267.4 (murine macrophages), *in vivo* efficacy against paracetamol-induced hepatotoxicity and pharmacokinetic study by oral route in Wistar rat.

Results: The formulation showed maximum entrapment (55%) for a lecithin–cholesterol ratio of 6:1. Comparative release profile of formulation was better than silymarin at pH 1.2 and pH 7.4. *In vitro* studies showed a better hepatoprotection efficacy for formulation (one and half times) and better prevention of ROS production (ten times) compared to silymarin. In *in vivo* model, paracetamol showed significant hepatotoxicity in Wistar rats assessed through LFT, antioxidant markers and inflammatory markers. The formulation was found more efficacious than silymarin suspension in protecting the liver against paracetamol toxicity and the associated inflammatory conditions. The liposomal formulation yielded a three and half fold higher bioavailability of silymarin as compared with silymarin suspension.

Conclusions: Incorporating the phytosomal form of silymarin in liposomal carrier system increased the oral bioavailability and showed better hepatoprotection and better anti-inflammatory effects compared with silymarin suspension.

Introduction

Silymarin, a hepatoprotective agent, obtained from single herb *Silybum marianum*, is widely used in the treatment of liver diseases. A mixture of flavolignan isomers, namely silybin, isosilybin, silydianin, silychristin is collectively expressed as silymarin [1]. Among these isomers, the most active component is silybin, which accounts for 60–70% of the total content of silymarin and is considered as the marker of silymarin [2].

Many experimental studies have proved the hepatoprotective activity of silymarin [3]. One of the major limitations of silymarin is poor oral-bioavailability. The oral absorption of silymarin is only about 23–47% [4], leading oral bioavailability to 0.73% [5]. Therefore, a higher dose of silymarin is required to improve therapeutic efficacy. The reasons suggested for its poor bioavailability includes the following: poor enteral absorption [6], instability in gastric environment [4] and poor solubility [4]. Thus, enhancement of bioavailability of silymarin is a challenging task. Although substantial advancement has been made in improving the bioavailability of silymarin through various dosage forms, little

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information is available on the measures adopted by researchers to make silymarin target specific to promote hepatocytes' regeneration and to prevent inflammation in liver [7].

The process of repair in the liver is largely by the regeneration of hepatocytes. However, inflammation in liver is one of the major problems associated with hepatocyte toxicity. If inflammation is not controlled sufficiently, the cellular phase of inflammation through macrophages (Kupffer cells) and fibroblast (stellate cells) promote fibrosis to replace dead cells [8]. Therefore, a formulation of silymarin that would target the liver in general and inflammation in particular would be beneficial over a formulation of silymarin that would just enhance the bioavailability of silymarin. In this context, the present study is aimed at developing a formulation of silymarin with the help of liposomal and phytosomal combination. This is based on the fact that phytosomal silymarin is more stable in the gastric environment [9] to enhance the bioavailability of silymarin while the liposomal silymarin is having the highest ability to get captured by macrophages, Kupffer cells and infiltrated WBC *viz.*, neutrophil, monocytes, *etc.* through phagocytosis process and modulate their actions [10]. This phenomenon makes silymarin in formulation to target inflammation.

Materials and methods

Materials

Triton-X 100, trypsin and dithiothreitol (DTT) were purchased from Himedia lab Pvt. Ltd. (Mumbai, India). Lecithin (Soya L-α-Phosphatidylcholine or SPC), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified eagle's medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS) and silymarin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in the study were of analytical grade.

Cell lines

Chang liver cells and RAW 264.7 were purchased from National Center for Cell Sciences (Pune, India).

Animals

Wistar rats of 4–6 weeks age, weighing 180–200 g were selected for study. The animals were acclimatized to for one week in controlled temperature and humidity conditions with 12:12 h light and dark cycle. The rats were fed standard food pellets and water *ad libitum*. The study was conducted after obtaining clearance from the Institutional Animal Ethics Committee of KMC.

Table 1
Optimization of lipid and drug ratio.

Formulation	SPC:cholesterol: drug (mg)	Molar ratio (SPC:C)	% Entrapment efficiency
L1	294:00:10	10:00	36.11
L2	255:15:10	09:01	32.84
L3	240: 15:10	08:01	18.69
L4	180:15:10	06:01	47.22
L5	240:30:10	04:01	32.22
L6	180:30:10	03:01	35.53
L7	180:77:10	1.5:1	11.06

SPC:cholesterol:drug ratio without freeze-drying at sonication of frequency – 80 Hz, time – 2 min and pulse – 4 s.

Preparation of liposomes

Preformulation studies

Silymarin was evaluated for its physicochemical interaction with lecithin (SPC) and cholesterol (C) at a ratio of 1:1:1 using differential scanning calorimetry (DSC).

Development of silymarin-liposomes

Liposomes were prepared by lipid film hydration method [11]. Silymarin (S) 10 mg, different quantities of SPC and cholesterol were taken into a round bottom flask and dissolved in methanol-chloroform mixture (1:9) (Tables 1 and 2). Later the solvent was evaporated under vacuum at 40 °C in a rotary evaporator to develop thin film. The solvent traces from film was removed by drying overnight in a vacuum desiccator. The film was hydrated with phosphate buffer saline (PBS, pH 7.4), containing varied amount of cryoprotectant (mannitol and sucrose) (Table 2) at 100 RPM and at 50 °C for 1 h to prepare a liposomal suspension. The liposome vesicle size was reduced under high-pressure homogenization at 20,000 psi for 5 cycles. The liposomes were kept overnight in deep freezer at −80 °C. The frozen liposomes were lyophilized at reduced pressure and stored at 4 °C in airtight containers for further experiments.

Physicochemical characteristics of liposomes

Particle size and zeta potential

Mean particle size, polydispersity index (PDI) and zeta potential of liposomes were determined by Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK) after suitable re-dispersion in water.

Drug entrapment efficiency in liposomes [12]

Liposomal suspension (1 ml) was centrifuged at 1000 rpm for 10 min to separate unentrapped particles. Supernatant was collected and again centrifuged at 64,000 × *g* at 4–8 °C for

Table 2
Optimization of various liposomal parameters for formulation L4.

Parameters		Preformulation of liposome (SPC:C 6:1)						Optimized
Freeze drying		NO	Yes	Yes	Yes	Yes	Yes	Yes
Cryoprotectant[a]		Nil	Nil	Mannitol 10%	Sucrose 10%	Mannitol 15%	Sucrose 15%	Sucrose 5%
Yield		98%	98%	97%	95%	68.75%	75.63%	98.5%
Particle size (nm)		100	701	1005	400	1205	405	329
Zeta potential		>−70 mV	>−70 mV	>−70 mV	>−70 mV	>−70 mV	>−70 mV	−70.5 mV
HPH[b] (Cycle, bars)		NA	10, 15,000	10, 15,000	10, 15,000	15, 20,000	10, 20,000	10, 20,000
% Entrapment	Sil A&B[*]	47.22	53.39	41.28	53.27	52.40	47.29	58.94
	Sil A[c]	NA	40.89	39.16	50.50	63.55	39.63	59.24
	Sil B[d]	NA	65.90	43.41	56.03	41.25	54.95	58.64

[*] Average of Silybin A and Silybin B
[a] Amount in w/v.
[b] High pressure homogenization.
[c] Silybin A.
[d] Silybin B.

60 min (Sigma centrifuge, SciQuip Ltd., UK). Supernatant was separated and stored for testing free drug content. To the pellet, 100 μl of 10% Triton X and 900 μl of methanol were added. The concentration of silymarin in supernatant and precipitate was analyzed by reversed phase high performance liquid chromatography (RP-HPLC). The HPLC column was Hibar® RP C-18, 4.6 mm × 250 mm from Merck, Darmstadt, Germany. The mobile phase methanol: water (50:50, pH 3.5) was pumped at a flow rate of 1.0 ml/min. The detection wavelength and the detection limit of silymarin adopted were 286 and 1 μg/ml respectively.

Morphology

Liposomes were suspended in water and particle shape was visualized by transmission electron microscope (TEM), TECNAI 200 Kv TEM from Sophisticated analytical instrument facility, All India Institute of Medical Sciences, New Delhi, India.

In vitro drug release [7]

The dissolution studies were carried out according to a dissolution test of China Pharmacopoeia (2005 edition, paddle method). The dissolution flasks were immersed in a water bath at 37 °C. The dissolution medium (pH 7.4 (PBS)) and pH 1.2 (HCl)) were continuously stirred at 100 rounds/min. Silymarin (77 mg), liposome formulations (equivalent to 77 mg of Silymarin) were added to the surface of the stirred dissolution medium at the beginning of the study. At different time intervals, 10 ml samples were withdrawn and filtered using 0.22 μm syringe filter; while 10 ml fresh medium were added into the flask. Forty-microliter aliquot of the resulting solution was injected into HPLC and detected at a wavelength of 286 nm; the concentration of silymarin was measured.

In vitro assessment

In vitro hepatoprotection

Silymarin and its liposome were compared for *in vitro* hepatoprotection of Chang liver cells against paracetamol(para)-induced heptotoxicity based on the principles of MTT cytotoxicity [13]. Cells were pretreated with various concentrations of testing samples in maintenance media for 24 h. Further, the cells were challenged with 100 μl of 50 mM of paracetamol for 24 h. The media were replaced with 100 μl of 1 mg/mL solution of MTT and incubated for 4 h. The formed blue colored product (formazan) was found to be proportional to the number of live cells.

$$\%\ \text{Viability} = 100 - \left[\frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100\right]$$

Nuclear staining study

Two thousand cells were seeded per well into 24-well plates with MEM containing 10% FBS. After 24 h, cells were treated with 7.5 μg/ml of the equivalent of silymarin and incubated for 24 h. The media were removed and the plate was washed with PBS (pH 7.4). Paracetamol (100 μl of 50 mM) in complete media was added and incubated for 24 h. Cells were fixed with ice-cold methanol for 20 min. Cells were washed with PBS again and 50 μl of Hoechst 33342 stain (2 μg/ml) was added to each well. The plate was incubated at 37 °C for 20 min. Finally, the plate was washed thrice with PBS and observed under a fluorescent microscope for morphonuclear changes [14].

Effect on antioxidant status of Chang liver cells

Chang liver cells (1×10^6 per ml) were seeded in six well plates for 24 h. Silymarin and its liposome were added at a concentration of 7.80 μg/ml after 24 h. Cells (except from control well) were stimulated with 50 mM paracetamol and incubated for 24 h. After 24 h, the media was removed and cells were lysed with cold buffer and used for estimation of glutathione (GSH) content [15] and lipid peroxidation (TBARS) levels [16].

Reactive oxygen species (ROS) inhibition assay

Murine macrophages ell line, RAW 264.7 cells were stimulated for ROS production by lipopolysaccharide (LPS) [17]. Silymarin and its liposome were studied using pretreatment in a concentration range of 31–250 μg/ml equivalent to silymarin.

In vivo assessment

In vivo hepatoprotection

Hepatotoxicity was induced in Wistar rat with a minor modification of the method of Zakaria et al. [18]. The toxic dose of paracetamol selected here was 2.75 g/kg p.o. instead of 3 g/kg used by Zakaria et al. Briefly, Wistar rats were randomly divided into four groups containing six animals each *viz.*, sham, paracetamol (para) control, silymarin, liposome treatment group. Caboxymethyl cellulose (CMC, 0.25%, w/v) was used as vehicle for the oral administration of silymarin, silymarin-liposomes and paracetamol. Silymarin and its liposomal formulations were administered for seven days at doses that had the contents of silymarin equivalent to 50 mg/kg. On day-6 of the study, paracetamol was administered once at a dose of 2.75 g/kg to induce hepatotoxicity. Forty-eight hours after the administration of paracetamol, blood was withdrawn and serum was separated to perform liver function tests (LFT). The effect was also studied on kidney by monitoring serum urea and serum creatinine level, and heart by monitoring creatine kinase level. The liver was dissected out, a part of liver was used for antioxidant and inflammatory marker study, and remaining part of liver was used for histopathology.

Estimation of antioxidant parameters

10% of liver homogenate was made in 150 mM KCl. Homogenate was centrifuged at 14,000 rpm for 15 min and used for the estimation of antioxidant parameters *viz.*, total thiols [15], glutathione (GSH) [15], lipid peroxidation [16], catalase [19] and super oxide dismutase (SOD) [20].

Estimation of inflammatory markers

Liver homogenate was used for estimation of interleukin-6 (IL-6) level (using kits from Krishgen Biosystem, Mumbai, India), nitrite level [21] and myeloperoxidase (MPO) level [22].

Bioavailability study in Wistar rats

The plasma concentration of silymarin (equivalent to combined silybin) was determined by RP-HPLC. The chromatographic conditions of HPLC were similar to analytical study. Twelve male Wistar rats were divided into two groups. Animals were fasted for 8 h. Silymarin and its liposomal formulation were administered orally after suspending them in 0.25%, w/v, CMC. The quantity of silymarin that was administered had silybin at a level of 200 mg/kg. Blood (250 μl) was withdrawn at the time points, *viz.*, 15 min, 30 min, 1 h, and 2 h intervals. To 100 μL of separated plasma, 5 μl of α-napthol was added as internal standard. The protein was precipitated by the addition of 300 μl chilled methanol: acetonitrile (50:50). The mixture was centrifuged at 14,000 rpm for 10 min. 40 μl of supernatant was injected into the column. The standard plot was made and the peak concentration, peak time, AUC, and other pharmacokinetic parameters were calculated by non-compartment modeling using WinNonline software, Pharsight Corporation, CA, USA.

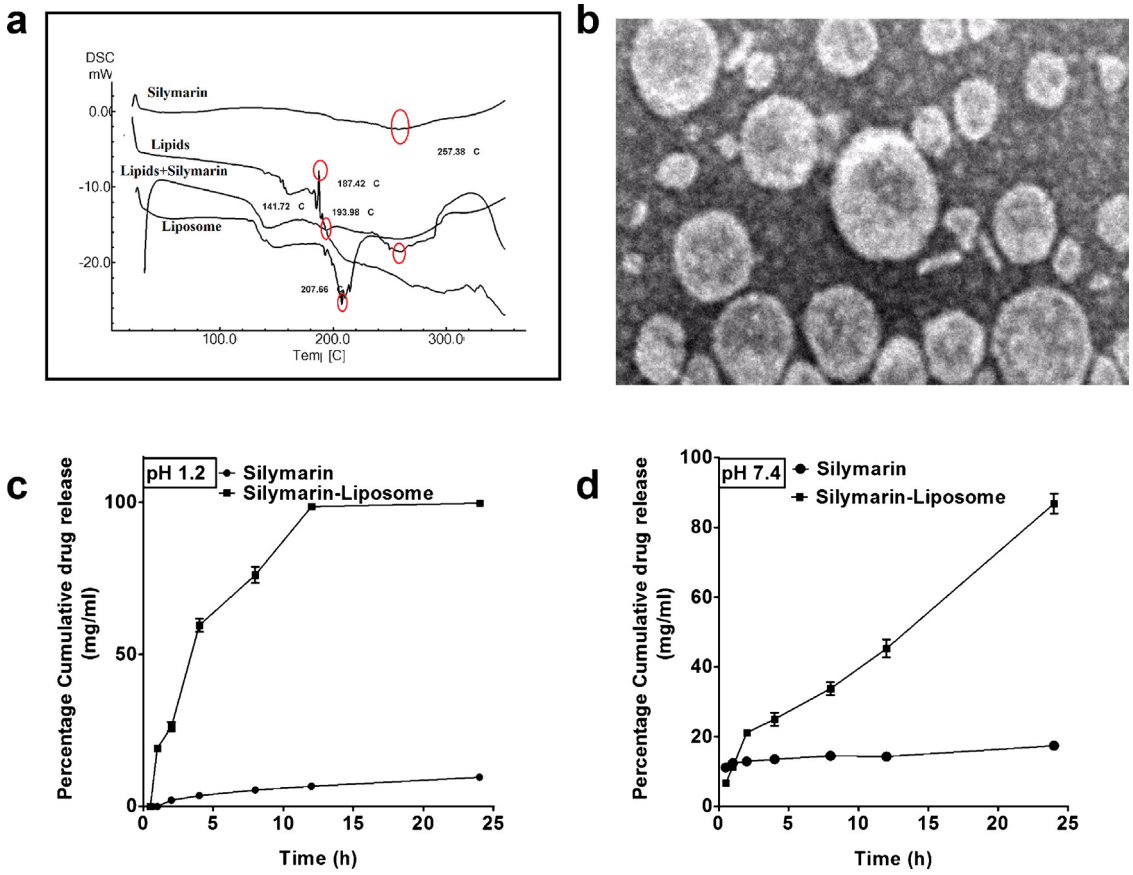

Fig. 1. Characterization of liposome. (a) DSC thermogram, (b) TEM image, (c) release study silymarin and its liposomes at pH 1.2, (d) release study silymarin and its liposomes at pH 7.4. All the values in (c) and (d), are mean ± SEM of three readings.

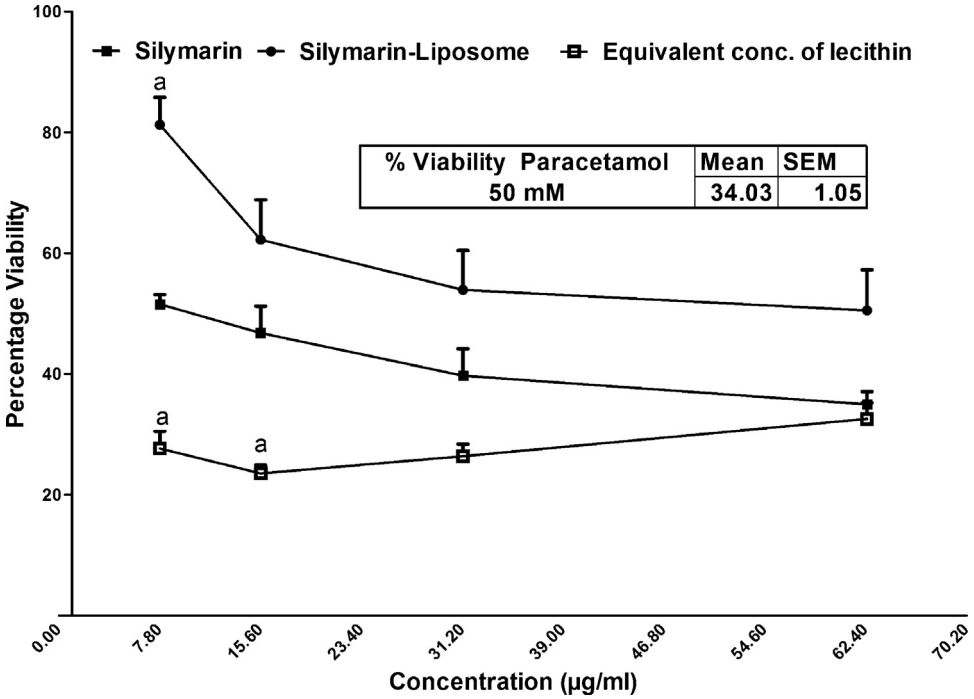

Fig. 2. *In vitro* hepatoprotection in paracetamol-induced toxicity against Chang liver cells. All the values are mean ± SEM of three tests in triplicate, $^{a}p < 0.05$ compared to silymarin.

Statistical analysis

Statistical analysis of the data was carried out by one-way ANOVA (Graph PAD Instat Software) followed by Tukey *post hoc* test. A value of $p < 0.05$ was considered to be significant.

Results

Preparation and characterization of silymarin-liposomes

Preformulation studies

Physical mixtures (silymarin and lipids) did not show any drug-lipid interactions. They were stable, physically and chemically as no changes were observed in the data of DSC (Fig. 1a).

Preparation of silymarin-liposomes

The encapsulation percentage of silymarin was found to be maximum for formulation containing SPC and cholesterol at molar ratio 6:1 (Table 1). Particle size obtained from sonication and high-pressure homogenizer (HPH) was compared. More homogeneous and uniform particles were obtained by HPH compared to sonication alone. A pressure of 20,000 psi for 5 cycles was found to be optimum for size reduction. Sucrose 5%, w/v, as cryoprotectant produced more hygroscopic and lesser size (below 500 nm) liposomes compared mannitol. This was selected as optimized formulation (Table 2).

Transmission electron microscopy for surface morphology

After slightly shaking in water, a monolayer formed. Particles appeared to be spherical in shape (Fig. 1b).

DSC analysis

DSC thermogram of silymarin showed an endothermic peak at 257.38 °C, while for liposomes, 207.66 °C. The shifts of endothermic peak of 40–50 °C in thermogram suggested a possible interaction of silymarin with lipid components and could account for enhanced entrapment (Fig. 1a).

In vitro drug release studies

Detection of silymarin was done based on the presence of amount of silybin A and silybin B. Silymarin used in the study had 44.9 and 57.31% of silybin A and silybin B, respectively. The dissolution amount was calculated on the average amount of silybin A and silybin B. The release rate of the liposomes was investigated by dissolution in gastric pH using HCl (pH 1.2) and intestinal pH, using phosphate buffer saline (pH 7.4). At pH 1.2, the liposomes showed an average cumulative drug release of $98.57 \pm 0.40\%$ whereas silymarin alone was found to be $6.65 \pm 0.38\%$ after 12 h (Fig. 1c). Conversely, at pH 7.4, the complete release ($86.80 \pm 2.87\%$) took place around 24 h later while for

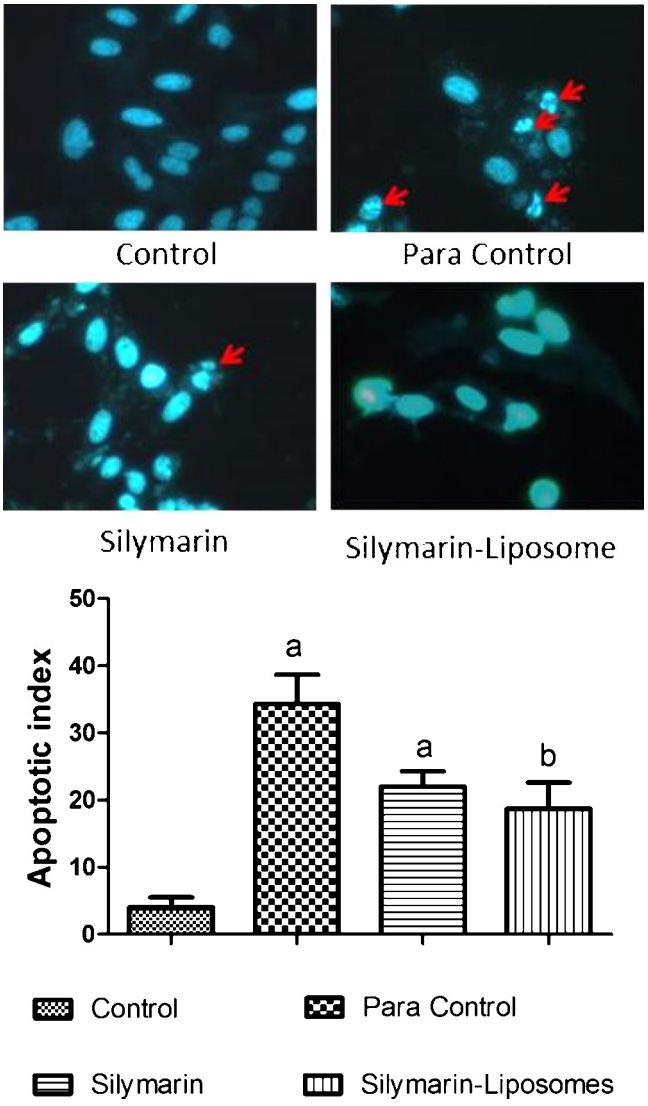

Fig. 3. Nuclear staining in paracetamol-induced toxicity on Chang liver cell line. Arrows indicating condensed nuclei and cytoplasm shrinkage; concentration of treatments: paracetamol – 50 mM, silymarin – 7.5 μg/ml, silymarin liposome – 7.5 μg/ml equivalent of silymarin. All the values are mean ± SEM of three tests in triplicate where $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to para control.

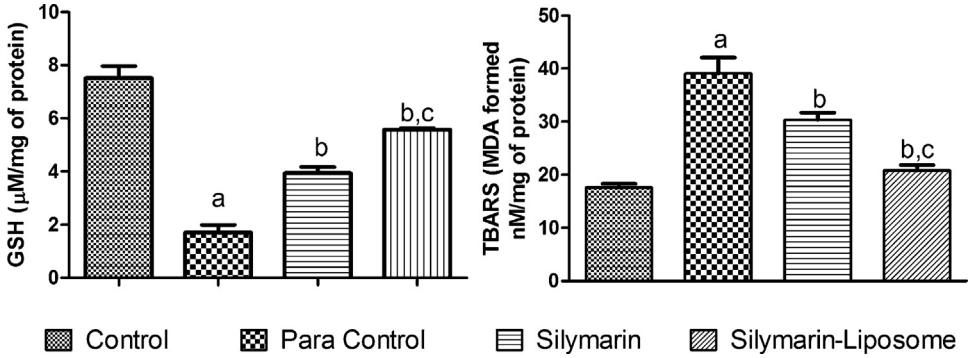

Fig. 4. Antioxidant parameter in paracetamol-induced toxicity in Chang liver cells. All the values are mean ± SEM of six animals where $^{a}p < 0.05$ compared to sham, $^{b}p < 0.05$ compared to para control, $^{c}p < 0.05$ compared to silymarin.

Table 3
Effect of treatments on ROS production by LPS on macrophage cell line.

Concentration (μg/ml)	Silymarin	Silymarin-liposome	Lecithin equivalent
	Percentage ROS inhibition		
	Mean ± SEM	Mean ± SEM	Mean ± SEM
31.25	−12.84 ± 4.06	62.61 ± 4.01	−12.73 ± 2.61
62.5	20.70 ± 0.09	83.98 ± 1.37	−22.93 ± 17.12
125	78.16 ± 1.68	89.91 ± 5.05	−126.60 ± 40.62
250	90.26 ± 1.05	91.24 ± 0.61	−158.42 ± 55.38

All the values are mean ± SEM of three tests in triplicate.

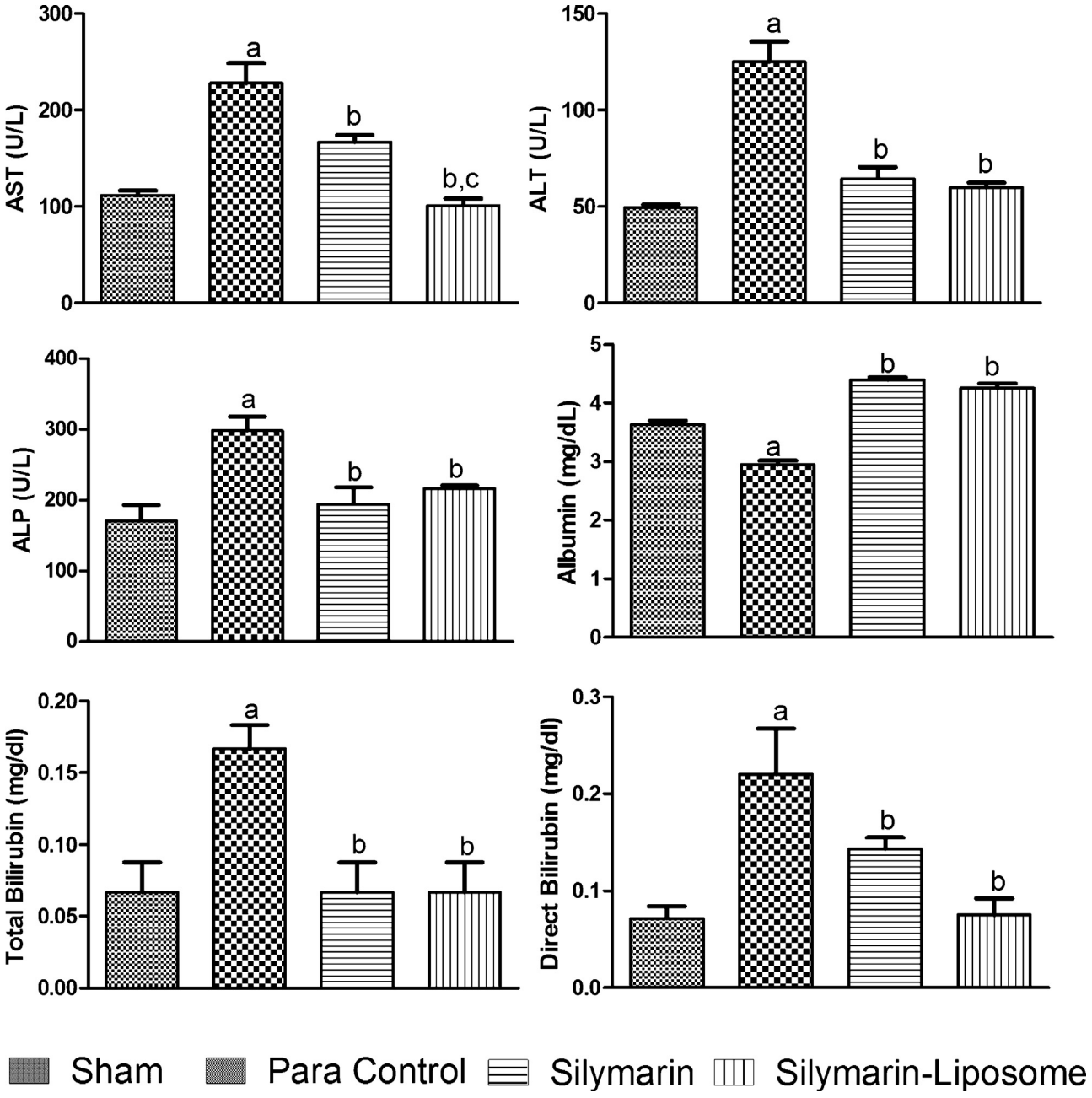

Fig. 5. LFT in paracetamol-induced toxicity in Wistar rats. All the values are mean ± SEM of six animals where [a] $p < 0.05$ compared to sham, [b] $p < 0.05$ compared to para control, [c] $p < 0.05$ compared to silymarin.

silymarin the release was only 17.42 ± 0.58% (Fig. 1d). Results showed that the formulation has increased the solubility of the drug.

In vitro studies

In vitro hepatoprotection

CTC_{50} (concentration at which 50% cells die) value of silymarin was found to be 151.2 μg/ml in Chang Liver cells. The dose of paracetamol to cause hepatotoxicity to Chang liver cells was 50 mM. The liposomal formulation of silymarin was found to be one and half-fold more active than silymarin in increasing percentage viability (Fig. 2).

Nuclear staining study

Paracetamol treatment produced nuclear condensation, and a disrupted membrane with cytoplasmic disintegration. The pre-treatments of cells with silymarin liposomes showed better prevention in these morphological changes associated with paracetamol treatment (Fig. 3).

Estimation of antioxidant parameters

Paracetamol treatment depleted GSH levels in Cells and a significant increase in TBARS level in the cells. Treatment with silymarin and liposomes increased the GSH content of cells and significantly lowered the TBARS level. The TBARS lowering effect was significantly more for silymarin-liposome compared with silymarin suspension (Fig. 4).

ROS inhibition assay

The liposomal preparation of silymarin was significantly more effective than silymarin in inhibiting LPS-stimulated ROS release from the RAW 264.7 murine macrophages (IC_{50}7.93 and 98.86 μg/ml respectively). Lecithin could not prevent ROS formation by RAW 264.7 (Table 3).

In vivo studies

In vivo hepatoprotection study

Effect on liver enzymes. The paracetamol challenge in control animals raised AST level about two folds compared to sham animals. Silymarin-liposomes treatment was significantly effective in preventing the paracetamol-induced rise in AST level, as it brought the levels back to normal unlike the silymarin *per se* treatment. Silymarin and its liposome pretreatment for 7 days significantly protected the liver against the paracetamol induced

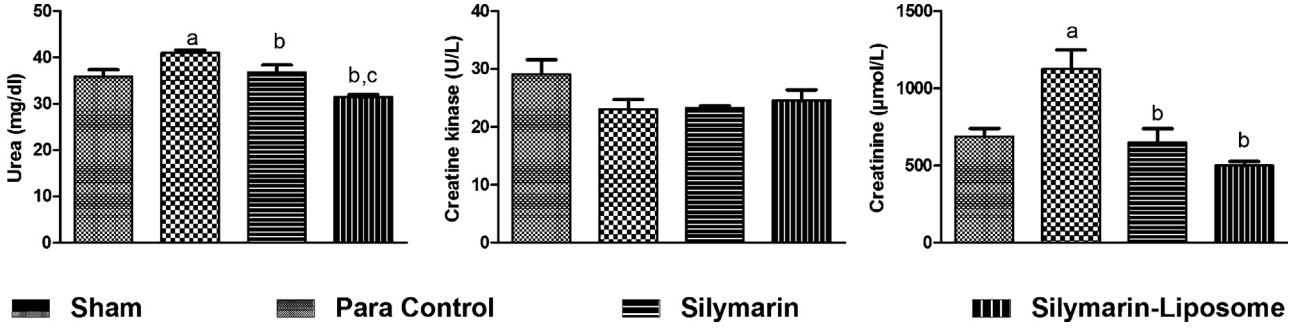

Fig. 6. Effect of silymarin and formulation on kidney and heart in paracetamol-induced toxicity in Wistar rats. All the values are mean ± SEM of six animals, where $^{a}p < 0.05$ compared to sham, $^{b}p < 0.05$ compared to para control, $^{c}p < 0.05$ compared to silymarin.

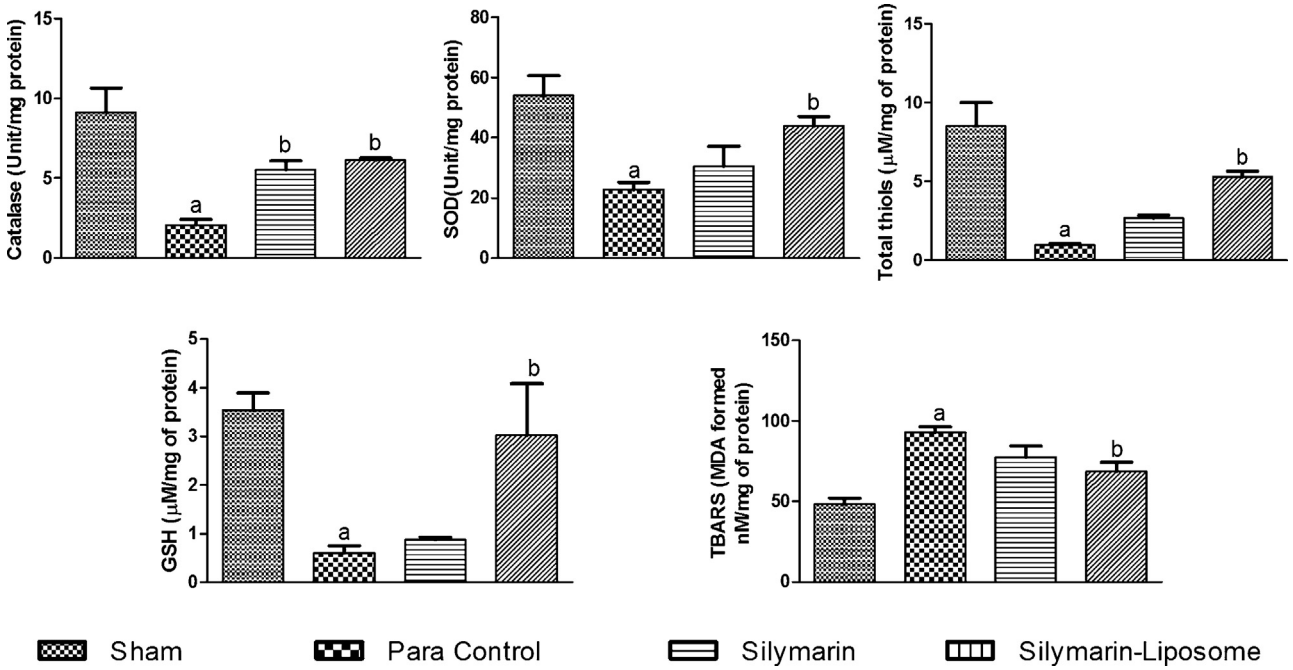

Fig. 7. Antioxidant parameter in paracetamol-induced toxicity in Wistar rats. All the values are mean ± SEM of six animals, where $^{a}p < 0.05$ compared to sham, $^{b}p < 0.05$ compared to para control, $^{c}p < 0.05$ compared to silymarin.

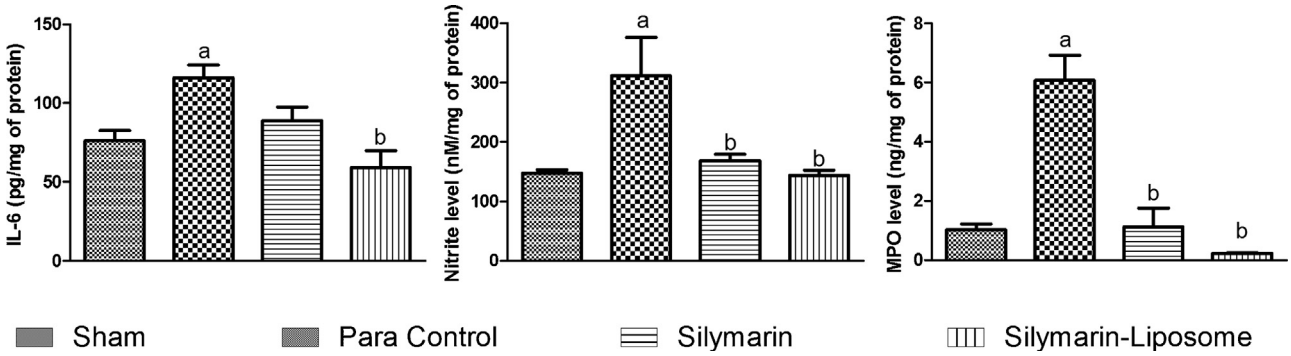

Fig. 8. Effects on Inflammatory markers. All the values are mean ± SEM of six animals, where $^{a}p < 0.05$ compared to sham, $^{b}p < 0.05$ compared to para control.

rise in the levels of ALT, total bilirubin and direct bilirubin parameters (Fig. 5).

Effect of silymarin and its liposomes on kidney and heart. Paracetamol administration also affected the kidney function (increased level of urea and creatinine in serum) of control rats. Silymarin and its liposome prevented these changes. Silymarin-liposomes showed better efficacy compared to silymarin alone in preventing the paracetamol-induced damage. No significant changes were observed in creatine kinase level (Fig. 6).

Estimation of endogenous antioxidant enzymes. A significant depletion in the antioxidant levels of liver was evidenced by a decline in the levels of catalase, SOD, total thiols and glutathione levels significantly in paracetamol treated animals as compared with the sham animals. The decline of antioxidant status correlated with a significant elevation of malonal dialdehyde (MDA) levels. Both, silymarin and silymarin-liposomes significantly reversed this effect (Fig. 7).

Estimation of inflammatory markers. Paracetamol toxicity increased IL-6 levels significantly in liver homogenate of control animals compared to sham animals. Silymarin-liposome significantly prevented the rise in IL-6 level and the activity was better than silymarin suspension (Fig. 8). Nitrite levels in liver homogenate of control animals increased significantly compared to sham animals. Liposomes significantly prevented the elevation in nitrite level and the activity was better than silymarin suspension (Fig. 8). Paracetamol treatment significantly raised the myeloperoxidase in liver homogenate to 6.074 ng/mg of protein while in control animals it was 1.015 ng/mg protein. Silymarin and its liposomes significantly prevented the elevation in MPO level (1.13 and 0.23 ng/mg of protein, respectively). The activity of silymarin-liposomes was comparatively better than silymarin suspension (Fig. 8).

Histopathology. Paracetamol intoxication showed fatty accumulation in hepatocyte. The central vein of the liver of paracetamol treated animals showed dilatation and congestion in sinusoids as compared with the sham animals. Treatment with silymarin at 50 mg/kg partially prevented fatty accumulation, while it failed in preventing the congestion in sinusoids and dilatation in the central vein. However, the silymarin-liposome pretreatment prevented all these changes of liver caused by paracetamol toxicity (Fig. 9).

Bioavailability studies in male Wistar rats. Silybin, the marker of silymarin, in plasma was separated by protein precipitation

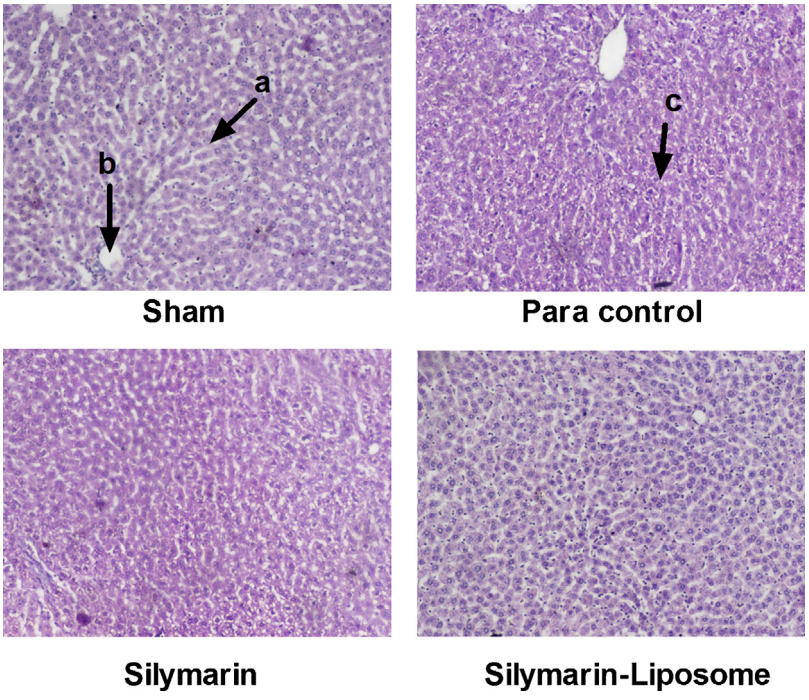

Fig. 9. Histology of liver in paracetamol-induced toxicity in Wistar rats. "a" indicates sinusoids, "b" indicates central vein, and "c" indicates fatty accumulation in hepatocyte.

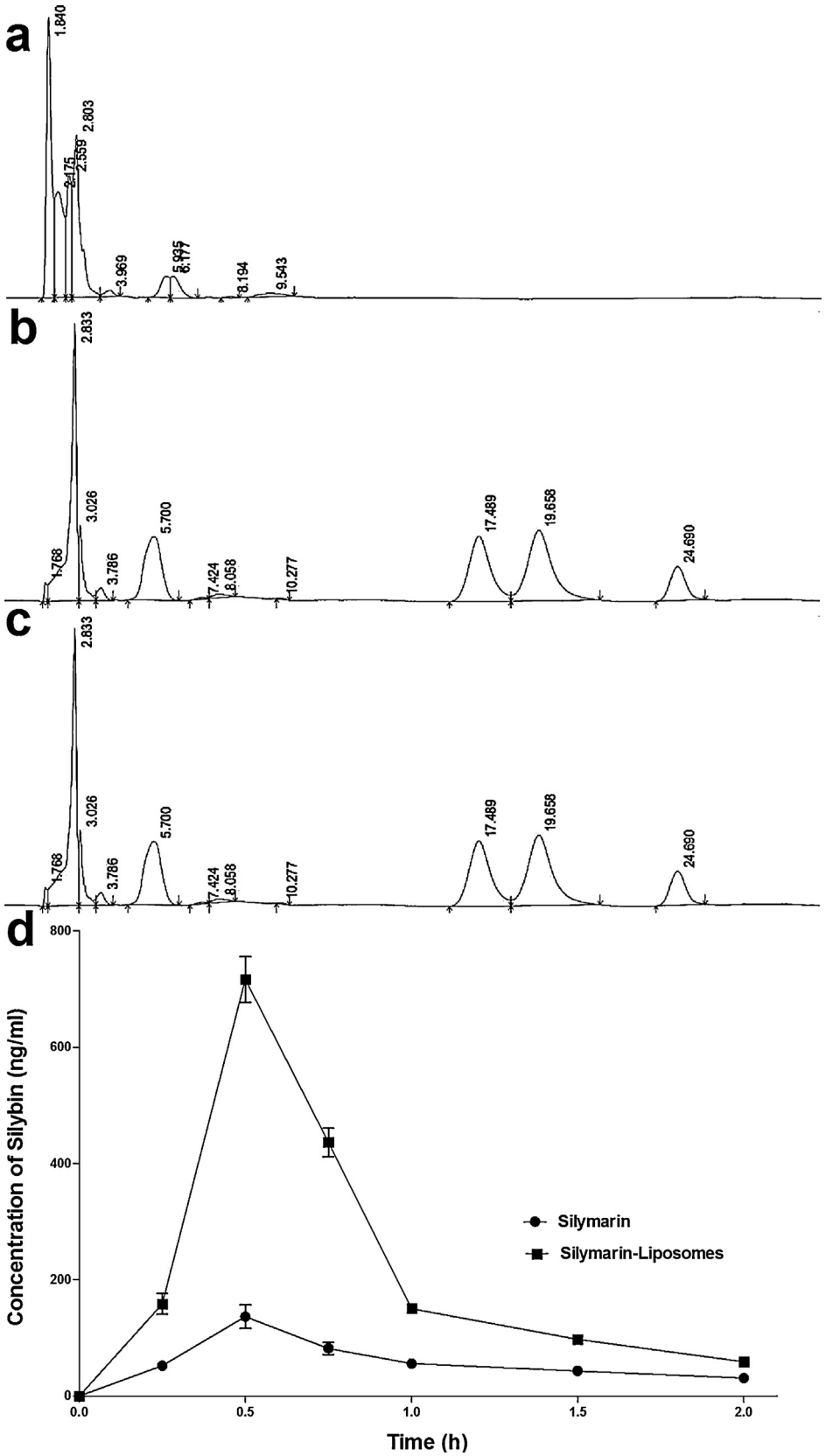

Fig. 10. Pharmacokinetic study. Typical chromatogram silymarin: (a) blank Wistar rat plasma, (b) spiked silybin and α-napthol, (c) a sample after oral administration of silymarin; (d) area under curve for silybin in silymarin and liposomes. All the values are mean ± SEM of six rats.

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

Pharmacokinetics parameters (unit)	Silymarin Mean ± SEM	Liposome Mean ± SEM
C_{max} (ng/ml)	136.52 ± 20.54	716.40 ± 42.85
t_{max} (h)	0.50 ± 0.0	0.50 ± 0
AUC_{0-2} (h*ng/ml)	115.20 ± 5.99	436.35 ± 21.59
AUC_{0-inf} (h*ng/ml)	158.83 ± 5.25	499.66 ± 23.40
Elimination rate constant (K_e) (1/h)	0.54 ± 0.06	1.04 ± 0.04
Half-life (h)	1.28 ± 0.17	0.67 ± 0.03
$AUMC_{0-t}$ (h*h*ng/ml)	103.04 ± 4.82	339.98 ± 14.82
$AUMC_{0-inf}$ (h*h*ng/ml)	258.89 ± 13.46	534.44 ± 19.97
Clearance (ml/h)	1266.02 ± 41.41	404.61 ± 18.59
Volume of distribution (V_d) (ml)	1946.93 ± 177.34	435.41 ± 24.8
Mean Residence $time_{0-t}$ (MRT) (h)	0.9 ± 0.009	0.78 ± 0.006
MRT_{0-inf} (h)	1.64 ± 0.09	1.07 ± 0.01

All the values are mean ± SEM of six animals.

method. Percentage recovery was 94–96%. Since silybin had two isomers, silybin A and silybin B, two peaks were obtained at 17.7 and 19.8 min. α-Napthol was used as internal standard with a recovery of 98%. The standard curve of silybin was prepared in the range of 30 ng/ml to 10 mg/ml, which was linear (r^2 0.9999). Average of the peak areas of both isomers was considered for analytical purpose. Typical chromatograms are displayed in Fig. 10a–c.

Concentration-time graph of silybin was plotted after oral administration of silymarin and its liposomal formulation (Fig. 10d). AUC_{0-2} of formulation was more than three and half times higher than silymarin suspension. The C_{max} obtained from the bioavailability study was 136.52 ± 20.54 ng/ml for silybin, with a t_{max} of 0.5 h. The C_{max} was increased by liposomal formulation to 716.40 ± 42.85 ng/ml without a change in t_{max} (Table 4).

Discussion

The optimized liposome of silymarin was spherical in shape and homogeneous in particle size distribution. Zeta potential of formulation (−70 mV) was found to be more than optimum for physical stability of the formulation [23]. DSC thermogram of liposomal formulation showed that there was an interaction between the lipid and drug, as the peak of silymarin shifted significantly by more than 40 °C. This interaction suggested the formation of phytosomes. The formulation had increased the solubility of silymarin at both tested pH compared to silymarin alone solubility.

Incubation of paracetamol with Chang liver cells resulted in reduction of cell viability while pretreatment with silymarin-liposomes significantly increased the percentage cell-viability as compared to silymarin suspension. The nuclear staining images also confirmed that the liposomes had a better role. The decreased level of GSH and high TBARS reflected the toxic effect of paracetamol, which was significantly prevented by silymarin-liposomes and, to a lesser extent, by silymarin suspension.

At a large acute dose, paracetamol causes hepatotoxicity by forming a large quantity of toxic metabolite N-acetyl-p-benzoquinone imine, which gets detoxified in the body by the formation of a conjugate, 3-glutathion-S-ylacetaminophen and results in depletion of endogenous antioxidants, mainly the GSH. Subsequently the non-metabolized toxic product covalently binds with protein and causes toxicity [24]. Similar findings were obtained in the present study. Paracetamol treatment depleted glutathione level almost by six folds, which was prevented by silymarin and its liposomes treatment. This sharp decline in glutathione was also associated with increased oxidative stress conditions like decline in antioxidant enzymes *viz.*, catalase, SOD and rise in MDA levels. Except SOD, other antioxidant parameters were significantly normalized by silymarin-liposomes to a greater degree than silymarin suspension.

As suggested by Pumford et al. [25], the toxic adduct with protein results in membrane damage and can be correlated with a rise in AST, ALT and mild increase in bilirubin levels. In the present study similar findings has been obtained. Silymarin and its liposomes significantly prevented these changes. The paracetamol intoxication resulted in centrilobular necrosis and ballooning in hepatocytes of liver parenchyma, which were minimized by pretreatment with silymarin and its liposomes. Paracetamol intoxication also resulted in kidney damage, marked by increased serum urea and creatinine levels [26]. The liposomes were significantly more effective than silymarin in normalizing the elevated urea levels, which indicated that the carrier systems might have increased the nephroprotective effect of silymarin together with hepatoprotective action.

Paracetamol intoxication increased the level of IL-6, which indicated inflammatory status of liver. Rise in the level of MPO indicated increased activity of inflammatory cells *viz.*, neutrophil, lymphocytes and Kupffer cells. This was also supported by a rise in nitrite level. Liposomes showed better activity in combating these inflammatory conditions as compared with silymarin suspension. This can be justified by the fact that Kupffer cells actively take up liposomes. Thus, silymarin in liposomal carrier system might have targeted inflammatory cells resulted in increased anti-inflammatory activity.

The above findings were supported by increased bioavailability of silybin from silymarin extract. C_{max} of liposomal silybin increased more than five times, with no change in t_{max}. More than threefold increase was observed in AUC level. Decrease in half-life and increase elimination rate constant can be justified by selective clearance of liposome by Kupffer cells.

Conclusion

Incorporating phytosomal form of silymarin in liposomal carrier system showed better *in vitro* and *in vivo* hepatoprotection besides showing better anti-inflammatory effects and improvement in histopathological changes as compared to silymarin suspension. These effects were further supported by increase in AUC and C_{max} of silybin by silymarin-liposomes compared to silymarin suspension.

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Conflict of interest

There authors declare there are no conflicts of interest.

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