4. Methodology

4.1. In vitro cytotoxicity studies for selected drug samples

4.1.1. Material

Cell line: Chang Liver cell line, Minimal Essential Media (MEM), fetal bovine serum (FBS) and MTT reagent from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. Tissue culture flasks, 96 well microculture plates from Tarsons Products Pvt. Ltd., Kolkata, India. Gentamicin, streptomycin, penicillin from KMC Hospital, Manipal, Karnataka, India.

4.1.2. Maintenance of cell lines

Chang liver cell line, procured from National Centre for Cell Science (NCCS), Pune, Maharashtra, India were grown in 25 cm² tissue culture flasks containing minimum essential medium (MEM) supplemented with 10% FBS, 1% L-glutamine and 50μg/ml gentamycin sulphate at 37°C in a CO₂ incubator in an atmosphere of humidified 5% CO₂ and 95% air. The cells were maintained by routine sub culturing in 25cm² tissue culture flasks.

4.1.3. Method for passaging the cells (Freshney, 2000)

All the reagents were brought to room temperature before use. Media was removed from the 80-90% confluent flasks by 10 ml serological pipette. Cells in T-75 flask were washed with 10 ml of PBS. Two milliliters of 0.1% trypsin EDTA was added to the flask. The flask was kept at 37°C in the CO₂ incubator for 2-3 min and was observed under microscope for detachment. Six milliliters growth medium was added to the flask for inhibition of trypsin action and resuspended properly by pipetting. The cell suspension was collected in 15 ml falcon tube, then centrifuged at 1200 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 3 ml of complete medium. Cells were counted, then 0.2-0.4 million cells were kept in T-25 flask for growing. The flasks were incubated in CO₂ incubator at 37 °C and the cells were periodically monitored for any morphological changes and contamination. After the formation of 80-90% confluent monolayer, the cells were further utilized.
4.1.4. Determination of cytotoxicity by MTT Assay (Eisenbrand et al., 2002)

The principle of cytotoxicity estimation is based on ability of the mitochondria of live cells to metabolize MTT to purple coloured formazan. The outcome of assay depends on both the number of cells present and the mitochondrial activity per cell. Mitochondrial enzyme succinate dehydrogenase cleaves tetrazolium salt 3-(4,5-dimethyl thiazole-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) into a purple coloured product (formazan). The number of cells is proportional to the extent of formazan production by the cells used.

![Figure 4.1. Principle of MTT assay](image)

4.1.4.1. Preparation of Test solutions

Ten milligrams of the drug samples were weighed and dissolved 10 ml of maintenance medium. These solutions were further diluted to obtain desired concentration.

Requirements

1. Confluent monolayer of Chang liver cells
2. PBS (7.4 pH)
3. 1% trypsin EDTA
4. Minimum Essential Medium (DMEM) with antibiotics
5. Fetal bovine serum
6. Eppendorf tubes  
7. Microtitre plate (96 well)  
8. Drug dilutions  
9. MTT (prepared in Hank’s Balanced Salt Solution (HBSS) without phenol red, 2mg/mL) (Sigma Chemicals)  
10. Dimethy sulphoxide (DMSO, Sigma chemicals)  
11. Microplate reader (ELISA Reader, Bio-Tek)  
12. Inverted Microscope (Nikon)  

4.1.4.2. Procedure  

a. The monolayer cell culture of Chang liver cells was detached by trypsinization. The cell count was adjusted to 5000 cells/100 µl using complete medium containing 10% new born calf serum.  
b. Hundred microliters of the diluted cell suspension was added to each well (approximately 5,000 cells) and incubated for 24h in CO₂ incubator.  
c. After 24h, the media was flicked off, and 100 µl of different drug concentrations were added to each well. The plates were then incubated at 37°C for 48h in CO₂ incubator. The plate was regularly monitored after every 24h.  
d. After a total 72h, 20 µl of 5mg/ml of MTT in PBS, was added to each well and kept back to CO₂ incubator for next 3h.  
e. Formation of formazan crystal was observed in microscope. If the crystal formation was not proper, incubation was continued for an hour more.  
f. Media was removed and 100 µl DMSO was added to each well. The plate was kept on shaker for 15 min. The absorbance was recorded ELISA reader at 540 nm.  
h. The percentage cytotoxicity was calculated by the following formula:

\[
\% \text{cytotoxicity} = \left[\frac{(\text{absorbance of control} - \text{absorbance of test})}{\text{absorbance of control}}\right] \times 100
\]

4.2. In vitro cytotoxicity studies for selected liver toxicants (Vijayan et al., 2003)  

In vitro cytotoxicity of selected liver toxicant viz., paracetamol, alcohol, D-galactosamine as assessed on Chang liver cell line by the MTT assay method as mentioned in section of 1.2. The exposure time of toxicant to cell line was 24h.
4.3. *In vitro* hepatoprotective activity of selected drugs against selected liver toxicants

4.3.1 *In vitro* hepatoprotective activity against D-galactosamine induced toxicity

Dose levels selected for the study were below the CTC₅₀ value for each drug sample.

a. 5000 cells/ 100 µL were adjusted using medium containing 10% fetal bovine serum.
b. 100 µL of suspended cells were added to each well of the 96 well microtitre plates.
c. After 24h, the media was flicked off, and 100 µl of different drug concentrations was added to each well. The plates were then incubated at 37°C for 24h in CO₂ incubator.
d. After 48h, 100 µl of D-galactosamine was added to each well except control well. The plates were then incubated at 37°C for 24h in a CO₂ atmosphere.
e. After 72h, media was flicked off. 20 µl of 5mg/ml of MTT prepared in PBS was added to each well and incubated in CO₂ incubator for next 3h.
f. Formation of formazan crystal was observed in microscope. If the crystal formation was not proper, plates were incubated for one more hour.
g. Media was removed by multichannel pipette and100 µl DMSO was added.
h. The plate was kept on shaker for 15 min. The absorbance was recorded by ELISA reader at 540 nm.
i. Percentage viability was calculated by the following formula:

\[
\% \text{ Viability} = 100 - \left[ \frac{(\text{absorbance of control} - \text{absorbance of test})}{\text{absorbance of control}} \right] \times 100
\]

4.3.2. *In vitro* hepatoprotective activity against alcohol induced toxicity

The procedure was the same as section 1.4 except step d, which was as follow:

d. After 48 h, 100 µl of 7.25% of alcohol in media was added to each well except control well. The plates were then incubated at 37°C for 24h in a CO₂ atmosphere.

Remaining steps were the same as section 1.6.1.

4.3.3. *In vitro* hepatoprotective activity against Paracetamol-induced toxicity

The procedure was the same as section 1.4 except step d, which was as follow:

d. After 48 h, the cells were challenged with 100 µl of 50mM paracetamol in media. The plates were then incubated at 37°C for further 24 hours in CO₂ incubator.

Remaining steps were the same as section 1.6.1.
4.4. **Nuclear morphological studies (Matzinger, 1991)**

The alteration or morphological changes in nucleus were observed by staining with a specific dye Hoechst 33342. This is a fluorescent dye, which reemits visible light upon absorbing ultraviolet light. Two thousand cells were seeded per well into 24-well plates with minimum essential medium containing 10% FBS. After 24h, cells were treated with 7.5 µg/ml of silymarin and equivalent to this for formulation. After 24h incubation, 100 µl of toxicant (Paracetamol 50mM, D-galactosamine 60mM, alcohol 7.25%) in complete media was added to each well except control well. The media was removed and the plate was washed with phosphate buffer saline (PBS, pH 7.4). Cells were fixed with ice-cold methanol for 20 min, washed with PBS again and 50 µl of Hoechst 33342 stain (2µg/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 morpho-nuclear changes.

4.5. **In vivo hepatoprotection study of selected herbal drugs**

**Materials** - Silymarin, Catechin, Lecithin (L-α-phosphatidyl choline from soya), D-galactosamine obtained from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. Alcohol obtained from S.D. Fine-Chem Ltd., Mumbai, Maharashtra, India.

**Animals**

Wistar rats of 6-8 weeks were maintained at Central Animal Research Facility, Manipal University, Manipal, Karnataka, India at temperature 23 ± 2°C, under controlled relative humidity (50±5), and 12:12 hour light and dark cycle were used in the study.(IAEC No.-IAEC/KMC/51/2009-2010)

4.5.1. **Effect of selected drugs in D-galactosamine-induced hepatotoxicity**

D-galactosamine-induced hepatotoxicity resembles viral-induced hepatotoxicity in its symptoms (Keppler et al., 1968). The animals were divided into five groups each containing six animals.

- **Group 1**  Sham (0.25% CMC)
- **Group 2**  D-Galactosamine control (400mg/kg *i.p.*)
- **Group 3**  Silymarin (50mg/kg)
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- Group 4  Catechin (50mg/kg)
- Group 5  Phyllanthin (50mg/kg)

The animals were administered drugs at 100 mg/kg, p.o. for 7 days. Vehicle used for the drug preparation was 0.25%w/v carboxy methylcellulose (CMC). On 7th day, D-galactosamine at 400mg/kg was injected intraperitoneally to all animals except animal of sham group (Shyamal et al., 2006). On 8th day, blood was withdrawn, serum was separated from blood by centrifuging at 5000 rpm for 5 min. Serum was used for estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) using Cobas C111 autoanalyser.

A portion of dissected liver was used for histo-pathological study and remaining part used for evaluation of antioxidant parameter.

Evaluation of antioxidant parameter

- Piece of liver was blot dried and homogenized in 150 mM KCl in cold condition and centrifuged in cooling centrifuge at 14000 rpm for 30 min to get the clear supernatant.
- The supernatant was used for estimation of following antioxidant parameters using standard protocol.

Assay for antioxidant Catalase (Aebi, 1984)

The catalase (CAT) activity was determined following the method of Aebi et al., 1984. 50 µl of tissue homogenate was added to a mixture of phosphate buffer (pH 7.0) and H$_2$O$_2$ (final O.D. between 3-5). The change in absorbance within a minute was recorded at 240 nm using UV spectrophotometer. The catalase activity was calculated and expressed as mMole/min/mg of protein.

Assay for antioxidant Super Oxide Dismutase (SOD) (Misra and Fridovich, 1972)

Tissue homogenate (50µL) 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 480nm (A$_0$-A$_{60}$). Change in absorbance was extrapolated to standard plot to find out the SOD activity, which was expressed in units/mg of protein.
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Assay for Glutathione (Hu, 1994)

The glutathione assay was performed by comparing the color formation due to derivative formation by DTNB. Equal volume of 5% trichloro acetic acid and the tissue homogenate were mixed and centrifuged to get a clear supernatant. The solution (500 µL of supernatant, 3 mL of PBS and 500 µL of DTNB) was incubated for 10 min at room temperature. The absorbance was recorded at 412 nm. The amount of glutathione was calculated by extrapolating on standard plot and expressed in µMole per mg of protein.

Assay of total thiols (Hu, 1994)

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 nMole/mg of protein.

Assay of lipid peroxidation (Konings and Drijver, 1979)

A mixture of 0.5 ml of tissue homogenate and 0.5 ml of thiobarbituric acid (TBA), trichloroacetic acid (TCA) and butylated hydroxytoluene (BHT) solution was heated at 90°C for 10 min. The mixture was centrifuged at 5000 rpm for 5 min and absorbance was taken at 530 nm. Lipid peroxidation was expressed as nanomoles of malonaldehyde formed per mg of tissue.

4.5.2. Effect of selected drugs in alcohol-induced hepatotoxicity

Chronic consumption of alcohol causes alcoholic liver disease. In the present study, animals were randomly divided into five groups each containing six animals.

- Group 1  Sham (vehicle- 0.25% CMC)
- Group 2  Control (Alcohol)
- Group 3  Silymarin (50mg/kg)
- Group 4  Catechin (50mg/kg)
- Group 5  Phyllanthin (50mg/kg)

Animals were orally administered ethanol at 15 ml/kg (45% v/v) two times a day for 30 days. Body weights were monitored on every alternate day. Treatment was started on day 31st and continued until day 45. Drugs were suspended in 0.25% w/v carboxy methylcellulose (CMC)
and administered orally. Control group received only 10ml/kg p.o. CMC (0.25% w/v). Alcohol was administered in drinking water at a concentration of 25% v/v. Total consumption of alcohol was monitored every day. On the 45th day blood was withdrawn and serum was separated by centrifuging it at 5000 RPM for 5 min. Serum was used for estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) using Cobas C111 autoanalyser.

A piece of dissected liver was used for histo-pathological study and the remaining part was used for evaluation of antioxidant parameter as mentioned in section 2.1.

Statistical analysis

Results were analyzed by one way ANOVA followed by Dunnett’s post hoc method of analysis (Graph PAD Instat Software). All the values are represented as mean±SEM (n=6), where *p<0.05, **p<0.01 and ***p<0.001 compared to control.

4.6. Formulation and development

4.6.1. Preformulation studies

Preformulation studies were carried out to determine the interaction between silymarin and lipids. Silymarin (CAS No. 65666-07-1), a yellow colored powder, 482.44 Da molecular weight, molecular formula of C_{25}H_{22}O_{10}, log P value of 2.4, melting point ranging 230 – 233°C, was mixed with L-α-phosphatidylcholine obtained from soya (SPC), cholesterol (C) and dicetyl phosphate, stearyl amine, 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (MPEG-DSPE-2000). The interaction between the lipids and silymarin was assessed through DSC and IR techniques and by observing a change in colour, melting point, transition temperature of the mixture and change of functional peak in IR spectrum in silymarin.

4.6.2. Development of Liposomal formulation of silymarin

Liposomes are the lipid based carrier systems, which are mainly used for targeting tissues and to improve bioavailability. Liposomes have been widely investigated for their properties as a model biomembrane and they are known to be taken up by liver cells (Bangham and Horne, 1964). Liposomes were prepared by the thin film hydration method. Phospholipid solution in organic solvent like chloroform forms a thin film after evaporation of solvent in a round bottom flask.
(RBF). Due to amphiphilic nature of phospholipid, it forms a bilayer spherical structure (liposome) upon hydration. Cholesterol in liposomal membrane structure provides rigidity and improves retention of solute (Tseng et al., 2007).

Liposomes were prepared by lipid film hydration method (Fang et al., 2006, Arumugam et al., 2008). Silymarin (S) 10mg, different quantities of phosphatidylcholine (SPC) and cholesterol (C) were taken into a round bottom and dissolved in methanol-chloroform mixture (1:9) (Table 5.3). Later the solvent was evaporated under vacuum at 40°C in a rotary evaporator to develop thin layer of lipids. The film was allowed to dry overnight in a vacuum desiccator to remove solvent traces. The lipid layer was hydrated with the help of phosphate buffer saline (PBS, pH 7.4), containing varied amount of cryoprotectant (Table 5.5), at 300 RPM and at 50°C for one hour to prepare a liposomal suspension of silymarin. The liposome vesicle size was reduced under high-pressure homogenization at 20000 psi for 5 cycles. The liposomes were kept overnight in a deep freezer at -80°C. The frozen liposomes were lyophilized at reduced pressure and stored at 4°C in airtight containers for further experiments.

**4.6.3. Formulation of optimized liposome**

Numerous trials were made to develop conventional liposomal delivery system with optimized parameters viz., smaller particle size (<1000 nm), homogenous particle size distribution (PDI<0.2) and entrapment efficiency (>30%). The following parameters were optimized:

- Selection of lipid
- Effect of molar ratio of lipids
- Selection of organic solvents
- Effect of cryoprotectant
- Effect of probe sonication
- Homogenization pressure and cycles

Effects of these parameters were studied on entrapment efficiency, particle size & its distribution, and zeta potential. The optimized formulations were further studied for XRD, DSC, TEM, *in vitro* release, stability, and *in vivo* studies in rats.
4.6.4. **Probe ultrasonication parameters:**

The influence of the amplitude and time of sonication on particle size reduction was studied by subjecting the different ratios of liposomal samples to probe ultrasonication with amplitude 40%, 60%, 80% each for periods of 2 min and 4 min. During this sonication, the temperature was maintained at 0°C using an ice bath. Effect of amplitude and time on particle size was evaluated by particle size analysis.

4.6.5. **Optimization of high-pressure homogenizer (HPH)**

Different cycles and different pressure were applied to achieve optimum particle size without damaging the formulation.

Optimization of amount of cryoprotectant - Amount of cryoprotectant was fixed based on the parameters of lyophilized liposomes viz., particle size, nature of powder.

4.7. **In vitro characterization of liposome**

4.7.1. **Entrapment efficiency (EE) by ultracentrifugation (Shivhare et al., 2009)**

Liposomal suspension (1 ml) was centrifuged at 1000 rpm for 10 min to separate unentrapped particle. Supernatant was collected and again centrifuged at 64,000 x g at 4-8°C for 30 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, 4.6×250mm from Merck KGaA, 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.

\[
\text{Encapsulation efficiency} = \left( \frac{\text{pellet}}{\text{supernatant} + \text{pellet}} \right) \times 100
\]

4.7.2. **Particle size and size distribution**

The mean particle size and its distribution are important parameters as they direct the saturation solubility, dissolution velocity, physical stability, and even biological performance of liposomes. The liposomes and their distribution are determined using Zeta Sizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) using a process called Dynamic Light Scattering (DLS).
Samples are examined for the particle size (PS), size distribution and poly dispersity index (PDI) at 25°C. In this technique, the time dependent fluctuations in the intensity of scattered light were measured. The time dependent intensity fluctuations are analyzed for the particles under Brownian motion, which enable to determine the diffusion coefficient of the particles, and converted them into the size distribution. This system is equipped with a 633nm, ic4mW Helium/Neon laser (Red laser) and it measure the Liposomal samples with the noninvasive backscatter technology at the detection angle of 173° (Malvern Instruments Ltd., Worcestershire, UK).

The results are represented as the average diameter of the liposomes suspension (Z-Average mean) with the PDI. The PS distribution was characterized using PDI, which is a measure for the width of the size distribution. The PDI of 0.0 (minimum reading) represents a homogenous particle population; while 1.0 (maximum reading) indicates a heterogeneous population.

4.7.3. **Zeta potential (ZP)**

ZP is the charge of the particle acquired in a particular medium. Its knowledge helps to assess the stability of the formulation during storage. Measurement of the ZP of the samples in Zetasizer (ZS) was done using a combination of Laser Doppler Velocitmetry (DLV) and Phase Analysis Light Scattering (PALS) by a patented technique called M-3 PALS to measure the particle electrophoretic mobility. In this technique, a voltage is applied across a pair of electrodes at either end of a cell which contains the particle suspension. Charged particles are attracted and move to the oppositely charged electrode and their velocity is measured and expressed in unit field strength as their electrophoretic mobility.

ZP of the liposomal suspensions was measured at 25°C. The ZP of particles more than (+) 30 mV and less than -30 mV are normally considered stable.

4.7.4. **Morphology of liposome by transmission electron microscopy**

The particle shape was visualized by transmission electron microscope, TECNAI 200 Kv TEM (Fei, Electron Optics) from Sophisticated analytical instrument facility, All India Institute of Medical Sciences (AIIMS), New Delhi, India. The liposomes were suspended in Diethylpyrocarbonate (DEPC) treated water (5 mg/ml).
4.7.5. Differential Scanning Calorimetry (DSC)

DSC measures the temperature dependent phase transition of the particle system. The curve of phase transition due to heat gives information on the enthalpy and entropy of liposomes. This is a sensitive tool for chemical composition and physical state (especially size) of liposomes (Biltonen and Lichtenberg, 1993). Liposomes were scanned in a range of temperature with 10°C increment per minute.

4.7.6. X-ray diffraction analysis

X-ray diffraction analysis of silymarin and its two liposomes namely conventional and PEGylated liposomes were performed in innovation center, Manipal Institute of Technology, Manipal, using X ray diffractometer (Rigaku, miniflex 600, Japan).

4.7.7. Short term stability studies (ICH guideline)

Optimized lyophilized formulations (PEGylated and conventional) were packed separately in amber coloured glass vials and placed in stability chamber at 25±2°C, 60±5%RH for a period of 3 months. Samples were checked for particle size, zeta potential and total drug content.

4.7.8. In vitro drug release (Yan-yu et al., 2006)

The release medium used was pH 1.2 acidic medium followed by Phosphate Buffer Saline pH 7.4 at 100 rpm USP-I (Basket method) at temp of 37°C. Silymarin (77mg), liposome formulations were 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. Twenty microliter aliquot of the resulting solution was injected into a HPLC and detected at a wavelength of 286 nm. The concentration of silymarin was measured.

4.8. In vitro hepatoprotection of developed formulation on Chang liver cell line against paracetamol, D-galactosamine and alcohol induced toxicity

The developed formulations were screened as per protocol mentioned in section 4.3.1.
4.9. Lipopolysaccharide-induced ROS release from RAW 264.7 cells

Lipopolysaccharide (LPS) is an activator of immune system. It induces a series of inflammatory reaction from local inflammatory reaction to septic shock. Inflammatory reaction to macrophages results in release of inflammatory mediators, which result in generation of reactive oxygen species. Reactive oxygen species (ROS) regulate various cellular functions such as DNA synthesis, transcription factor activation, gene expression, and proliferation.

**Method** (Mathew et al., 2013)

Cells (5×10^4/well) were seeded in a black 96-well plate. It was pretreated with various concentrations of treatment for 1h. Cells of each well were exposed to LPS (10 µg/ml), except the control well and incubated for 20h. Supernatant was replaced with DCFH-DA (100 µM,) in a microtitre plate. DCFH-DA is a cell-permeable non-fluorescent substance which undergoes intracellular oxidation and reacts with ROS to produce the highly fluorescent dichlorofluorescein. After one hour of incubation the wells were washed with freshly prepared Hank’s balanced salt solution and intensity of fluorescence was measured by fluorescence microplate reader (FLx800, Biotek Instrument Inc., Winooski, VT, USA) at an excitation wavelength 485nm and emission wavelength 530nm. The experiment was performed in duplicate.

4.10. *In vivo* hepatoprotection of developed formulation

4.10.1. *In vivo* hepatoprotection of developed formulation in Paracetamol induced hepatotoxicity model in Wistar rats

Animals weighing 150-180 g were randomized into six animals in five groups as per body weight. Animals were pretreated with the treatment as mentioned below for 7 days.

- **Group 1**  Sham (0.25% w/v CMC, 10ml/kg)
- **Group 2**  Control (paracetamol 2.75g/kg p.o.)
- **Group 3**  Silymarin (50mg/kg)
- **Group 4**  Conventional liposome (50mg/kg equivalent to silymarin)
- **Group 5**  PEGylated liposome (50mg/kg equivalent to silymarin)

The hepatotoxicity was induced in Wistar rat with a minor modification as per the method of Zakaria et al., 2011 (Zakaria et al., 2011). The toxic dose of paracetamol selected here was 2.75
g/kg p.o. compared to 3 g/kg used by Zakaria et al. 0.25% caboxymethyl cellulose (CMC) 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-6th of the study paracetamol was administered at once at a dose of 2.75 g/kg to induce hepatotoxicity. Forty-eight hours after the administration of paracetamol (the liver toxicant), blood was withdrawn and serum was separated from the blood by centrifuging it at 5000 RPM for 5 min.

Serum was used for estimation of various parameters by Cobas C111 autoanalyser viz., Liver function tests viz., aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin and albumin. The effect was also studied on kidney functions viz., serum urea and serum creatinine level and heart viz., creatinine kinase level.

The liver was dissected out and a part of liver was used for histo-pathological study and remaining part was homogenized and used for evaluation of antioxidant status through parameters viz., catalase, superoxide dismutase, total thiols lipid peroxidation and glutathione levels (as mentioned in section 3.1) and inflammatory parameters viz., MPO, Nitrite and IL-6 levels.

**4.10.2. *In vivo* hepatoprotection of developed formulation D-galactosamine induced hepatotoxicity model in Wistar rats**

Animals weighing 150-180 g were randomized into six animals in five groups as per body weight. Animals were pretreated with the treatment as mentioned below for 7 day.

- **Group 1** Sham (0.25% w/v CMC, 10ml/kg)
- **Group 2** Control (D-galactosamine, 400 mg/kg i.p.)
- **Group 3** Silymarin (50mg/kg)
- **Group 4** Conventional liposome (50mg/kg equivalent to silymarin)
- **Group 5** PEGylated liposome (50mg/kg equivalent to silymarin)

Drugs were administered in 0.25% carboxy methylcellulose (CMC) orally. The control group received only 10ml/kg of 0.25% w/v CMC. D-galactosamine at 400mg/kg dose was injected
intrap eritoneally to animals of group 2 to group 5 on 7th day. After 24 h of D-galactosamine injection, blood was withdrawn and serum was separated from the blood by centrifuging it at 5000 RPM for 5 min. Various parameters were evaluated as mentioned in section 6.1.

4.10.3. In vivo hepatoprotection of developed formulation in alcohol induced hepatotoxicity model in Wistar rats

Wistar rats weighing 150-180 g were divided into six animals in five groups:

- Group 1  Sham (0.25% w/v CMC, 10ml/kg)
- Group 2  Control (Alcohol)
- Group 3  Silymarin (50mg/kg)
- Group 4  Conventional liposome (50mg/kg equivalent to silymarin)
- Group 5  PEGylated liposome (50mg/kg equivalent to silymarin)

The animals were administered 15 ml/kg of ethanol (45% v/v) p.o. two times a day for 30 days. Treatment was started on day 31st and continued until day 45. Silymarin and its liposomal formulations were administered for seven days at doses that had the contents of silymarin equivalent to 50 mg/kg. CMC (0.25% w/v) was used as vehicle for the oral administration of silymarin, silymarin-liposomes. Silymarin and its liposomes were administered as suspension in 0.25% w/v CMC. A change was made in alcohol dosing, in place of twice a day, alcohol were administered in drinking water at a concentration of 25% v/v. On 45th day, blood was withdrawn in two aliquot, one with disodium EDTA for hematological profile using ELMACK Cell counter, another without EDTA for serum separation. Serum was separated from the blood by centrifuging it at 5000 RPM for 5 min. Various parameters were evaluated as mentioned in section 6.1.

**Statistical analysis**

Results were analyzed by one way ANOVA followed by Tukey’s post hoc method of analysis (Graph PAD Instat Software). Where a p<0.05 compared to sham, b p<0.05 compared to control and c p<0.05 compared to silymarin.
4.11. Pharmacokinetic study

Conventional liposomes showed better hepatoprotective action compared to PEGylated liposomes. Thus, conventional liposomes were selected for pharmacokinetic study by oral route. The pharmacokinetic parameters of formulation were compared with free drug in normal animals and in animals of same condition as used in alcohol induced hepatitis model.

The plasma concentration of silymarin (equivalent to silybin) was determined by HPLC. The chromatographic conditions were same as section 2.5.3. Twelve Wistar rats were divided into two groups. Animals were fasted for 8h. Silymarin and its liposomal formulation were administered orally after suspending them in 0.25% carboxy methylcellulose to maintain uniformity. The quantity of silymarin that was administered had silybin at a level of 200 mg/kg. The blood (250µl) was withdrawn at the time points viz., 15 min, 30 min, 1h, 1.5 h, and 2h 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) mixture. The mixture was centrifuged at 14000 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 WinNonlin software (Pharsight, Certara L.P., Sunnyvale, CA, USA).