6. Discussion

Silymarin, phyllanthin, lecithin, catechin, glycyrrhizin, picroside, etc. are the leading herbal drugs used for hepatoprotective action. Among these, four drugs namely, silymarin, phyllanthin, catechin and lecithin were selected for the proposed study. These herbal drugs suffer from poor pharmacokinetic profile viz., poor absorption, poor miscibility with oils and other lipids which make them less efficacious (Kidd, 2009, Manach et al., 2004). These problems can be overcome by lipid based carrier systems such as liposomes and phytosomes. This assumption is based on the fact that these carrier systems possess an amphiphilic constituent viz., lecithin, which is a known hepatoprotective agent. Amphiphilic nature of lecithin (solubility in water and oil) increases solubility of the drug molecules in the membrane and thus enhances the oral absorption of flavonoids. However, in gastric environment, while liposomes are unstable, the phytosomes are stable. On the other hand, liposomes are readily cleared from the circulation by monocyte and macrophages by phagocytosis (Kelly et al., 2010) and are targeted to the liver cells as well as inflammatory macrophages in a better way than phytosomes (Kovelamudi, 2012). Thus, the combination of these two carrier systems was considered for better advantages.

The selected drugs viz., silymarin, catechin, phyllanthin, and lecithin were compared for their efficacy in in vitro hepatoprotective models against paracetamol, D-galactosamine and alcohol-induced toxicity on Chang liver cell line. The initial dose selected for screening of in vitro hepatoprotective action of these drugs was 62.5µg/ml, which was close to half of the CTC50 value of the herbal drug with high cytotoxicity (silymarin: CTC50 151.2 ± 1.46). The other three dose levels were 7.82, 15.6, and 31.25µg/ml for the selected drugs. The dose levels for toxicants; paracetamol, D-galactosamine, and alcohol were 50mM, 60mM and 7.25%v/v, at which cytotoxicity was more than 50%. These toxicants represent three major categories of toxicities. Paracetamol-induced hepatotoxicity represents drug-induced acute liver disease model, D-galactosamine-induced toxicity is a viral pattern of liver toxicity model and alcohol induced toxicity mimics a chronic toxicity model. From the study, we found that silymarin showed maximum protection among the selected drugs against D-galactosamine, paracetamol and alcohol induced toxicities. The efficacy was also comparable in the in-vivo hepatotoxicity models, namely an acute toxicity model [D-galactosamine induced] and a chronic model [alcohol induced]. Silymarin showed comparatively better hepatoprotective profile in all toxicity models.
In our previous study, silymarin was found to be a better hepato-protective agent compared to catechin in D-galactosamine-induced acute hepatotoxicity model (Raj et al., 2010). Thus, we selected silymarin to improve its bioavailability and targetability to hepatocyte and inflammatory cells. Silymarin is a well-known hepatoprotective agent and acts through boosting the antioxidant defense of liver. The reasons for its low bioavailability includes poor enteral absorption (Comoglio et al., 1995), instability in gastric environment (Blumenthal et al., 2000), poor solubility (Blumenthal et al., 2000) and high excretion rate. Various formulations have been tried for increasing bioavailability of silymarin viz., salt forms (2 fold) (Madaus et al., 1976) self-microemulsifying drug-delivery system (4 fold) (Woo et al., 2007), solid dispersions (5 fold) (Chen et al., 2005), inclusion complexes of silybin with cyclodextrin (6 fold) (Valcavi et al., 1993), complexation with phospholipids (10 fold) (Vailati et al., 1993) and a significant improvement of bioavailability has been also achieved with pro-liposomes (Yan-yu et al., 2006) and hybrid liposomes (El-Samaligy et al., 2006). Although substantial advancement has been made in improving the bioavailability of silymarin through various dosage forms, little information is available on the measures adopted by the researchers to make silymarin target specific to promote hepatocyte regeneration and to contain inflammation in liver. Phytosomal silymarin was found to be stable in the gastric environment (Singha et al., 2011). On the other hand, liposomes are known for their ability to target immune cells (Bankey et al., 1995, Moghimi et al., 2012). Therefore, the formulation developed (the phytoliposomal-silymarin) was thought to be more specific to control the inflammatory phase of liver damage.

Silymarin, obtained from Sigma Aldrich Chemicals, USA, was a mixture of flavolignan isomers, namely silybin, isosilybin, silydianin, silychristin. In the present study, the most active component i.e., silybin was considered as the marker of silymarin (Pade, 2007) for the development of the proposed carrier system. The encapsulation percentage of silymarin in the formulation was found to be maximum at a molar ratio of 6:1. 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 and IR spectral data. The DSC thermogram of the liposomal formulation showed interaction between lipid and drug, as the peak of silymarin shifted significantly. The produced liposomes were found to be spherical, as seen in TEM image. The particle size of liposomes was in nanometer range except SA-liposomes, which was in micrometer range. Small size liposomes have longer circulation half-
life and they are slowly cleared by the RES system. Thus it might increase the chances of more interaction with hepatocyte (Sharma and Sharma, 1997). 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 +30mv to -30mv) results in physical instability of nano-formulation (AVERINENI et al., 2012). In the present study, zeta potential of the formulation was found to be in a stable range (<-30mv), which suggested physical stability of the formulation. The release rates of these liposomes were investigated by dissolution in gastric pH using HCl (pH 1.2) and intestinal pH using phosphate buffer saline (pH 7.4). Detection of silymarin was done based on the presence of the 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 based on the average amounts of silybin A and silybin B. The percentage cumulative drug release was significantly high for the conventional liposome in pH conditions of 1.2 &7.4, although complete release of silymarin in pH 7.4 occurred in 24h. 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 7.4. However, silymarin (alone) exhibited the lowest solubility in both pH conditions. This finding suggested that the formulation had increased the solubility of silymarin.

Liver damage associated with paracetamol consumption results in depletion of glutathione and antioxidant defense. Hence, using antioxidants to ameliorate the liver toxicity is a rational approach. This approach has been successful in various in vitro and in vivo hepatotoxicity models (Raj et al., 2010, Raj et al., 2011) which suggests that pretreatment of hepatocytes with flavonoids increases hepatoprotection against various toxins (Raj et al., 2008). The protective role of silymarin and its liposomes were examined on Chang liver cells. Incubation of paracetamol with the cells resulted in reduction of its viability. Pretreatment by all liposomes increased the percentage viability of Chang liver cells compared to silymarin alone. Conventional liposomes and PEGylated liposomes were found to be better among all four developed liposomes in increasing the percentage viability. A significant rise in the percentage viability at the tested concentrations of conventional liposomes as compared to silymarin alone proved that the tested formulations were effectively hepato-protective agents. Similarly
PEGylated liposomes were also effective in elevating the viability of Chang liver cells. The liposomes at higher concentrations were not able to offer protection, possibly due to enhanced toxicity of silymarin. The nuclear staining images also confirmed that the conventional and PEGylated liposomes had a better role in protecting the Chang liver cells against the toxicants tested as compared to silymarin alone.

Inhibition of ROS formation by silymarin and its liposomes was studied on RAW264.7 cell line. Conventional and PEGylated liposomes of silymarin showed potent inhibitory activity on LPS induced-ROS formation by RAW 264.7 cells as compared to silymarin per se. Lecithin could not prevent ROS formation in RAW 264.7 macrophages, even at the high dose tested. Based on the findings of these studies the conventional and PEGylated liposomes were selected for assessing their target specific efficacies in vivo in the animal models of hepatotoxicity viz., paracetamol, D-galactosamine and alcohol-induced hepatotoxicity in Wistar rats.

Paracetamol at a large acute dose (2.75g/kg p.o.) caused hepatotoxicity. It is known to cause toxicity by forming a large quantity of toxic metabolite N-acetyl-p-benzoquinone imine. The toxic product is detoxified in the body by the formation of a conjugate, 3-glutathion-S-ylacetaminophen (Jollow et al., 1974, Mitchell et al., 1973a), which results in the depletion of endogenous antioxidants mainly GSH. Subsequently the non-metabolized toxic product covalently binds with proteins and causes toxicity (Cohen and Khairallah, 1997). Similar findings were observed in the present study. Paracetamol treatment depleted glutathione level almost by six fold. Silymarin and its liposomes prevented the decline in glutathione level. A sharp decline in reduction of glutathione was also associated with increased oxidative stress, observed by decline in antioxidant enzymes viz., catalase, SOD and increased presence of free radical in terms of raised MDA levels. Antioxidant parameters were significantly preserved by silymarin liposomes and were found to be better than silymarin suspension. As suggested by Pumford et al., 1989 (Pumford et al., 1989), 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, paracetamol toxicity showed a rise in AST, ALT and total bilirubin levels. Silymarin and its liposomes significantly prevented these changes. The serum albumin levels are the markers of the biosynthetic function of liver. The levels of albumin decreased significantly due to paracetamol toxicity. Pretreatment with silymarin and its formulation prevented the fall in
serum albumin level and thus reflected liver protection. The paracetamol intoxication results in centri-lobular necrosis and ballooning in hepatocyte of liver parenchyma. Similar findings were observed in histopathology of paracetamol intoxicated group. These problems were minimized by silymarin and its liposomal pretreatment. Paracetamol toxicity is associated with fatty changes and infiltration of inflammatory cells viz., lymphocytes, and neutrophils. It is marked by a rise in level of inflammatory cytokines viz., IL-6, IL-2, TNF-α etc in liver tissue (Hinson et al., 2010). Increase in neutrophil infiltration and macrophage activity namely Kupffer cells, results in increased level of myeloperoxidase and rise in tissue nitrite level. Similar findings were observed in liver homogenate. Paracetamol intoxication increased the level of IL-6, which indicated inflammatory status of liver. Rise in the level of MPO indicated increased a rise in activity of inflammatory cells viz., neutrophil, lymphocytes, and Kupffer cells, supported by a rise in nitrite level. Both liposomes (the conventional and PEGylated) showed good activity in combating the inflammatory conditions compared to silymarin suspension. This can be explained by the fact that Kupffer cells have the ability to actively take up liposomes. Thus, silymarin from liposomal carrier system could have targeted the inflammatory cells to result in an increased anti-inflammatory activity. Paracetamol also caused kidney damage, marked by increased serum level of urea and creatinine (Cobden et al., 1982), which was significantly lowered by liposomes and silymarin (Nirala and Bhadauria, 2008). 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.

D-galactosamine induced hepatotoxicity resembles the viral pattern of hepatitis. It leads to induction of hepatic injury (extensive necrosis) in experimental animals. The metabolism of D-galactosamine (UDP-Galactosamine) interferes with the nucleic acid metabolism (Decker and Keppler, 1974). UDP-Galactosamine is same like UDP-glucose, which is produced by galactose metabolism. UDP-glucose is a product of galactose metabolism, which serves as an uridylate donor in the uridylyl transferase reaction. Thus, it leads to a remarkable decrease in the concentration of UDP-gluconate and results in a decrease in uridylylate biosynthesis which further causes a decline in RNA synthesis (Zimmerman, 1978). Similarly, this pattern of hepatotoxicity results in more than 10 times increase in AST, ALT levels. A similar finding was observed when D-galactosamine was injected intraperitoneally. There was a slight elevation in total and direct
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bilirubin while a decrease was seen in albumin, which shows altered synthetic function of liver. These serum parameters established hepatotoxicity in Wistar rats. Silymarin and its liposomal formulations showed improvement in these parameters. Conventional liposomes showed better prevention in all LFT parameters. No change was observed in urea, creatinine and creatinine kinase which suggested that D-galactosamine had not affected these parameters. Seven daily dosages of liposomal formulations did not cause any elevation in these parameters. Thus, it was confirmed that the formulations might be safe on kidney and heart.

D-galactosamine affects liver by affecting galactose pathway through altering the nuclear functions. These disturbances result in decrease in antioxidant enzymes synthesis. D-galactosamine injection decreased antioxidant levels viz, catalase, SOD, total thiols, GSH and increased free radical levels. Both liposomes prevented D-galactosamine induced damage to antioxidants.

Chronic alcohol ingestion produced liver damage (TSUKAMOTO and LU, 2001), marked by raised levels of the LFT parameters viz., AST, ALT, total bilirubin significantly (p<0.05) in 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 alcohol control group, which indicated that the synthetic function of liver was compromised. Chronic administration of alcohol is known to produce oxidative stress to cell (TSUKAMOTO and LU, 2001). 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 (TSUKAMOTO and LU, 2001). A similar finding has been observed in our study. The depleted GSH, total thiols, catalase and SOD levels were observed in 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 p450-2E1(CYP2E1) of mitochondria leading to the initiation and propagation of lipid peroxidation (TSUKAMOTO and LU, 2001). Finding in this study indicated a similar condition; alcohol intoxication increased the TBARS level (measured in 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., malonaldehyde, forms immunogenic adduct with transport protein, which in turn initiates the humoral and cellular immune responses (TSUKAMOTO and LU, 2001). 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. Alcohol intoxication is known to cause dehydration (Madeira et al., 1993) and also affects blood picture, which is accompanied by decreased WBC count. Chronic alcohol consumption in rats increased Hb and RBC count, which might be due to dehydration. It also increased MCV (TØNNESEN et al., 1986) and decreased platelet count (Renaud and Ruf, 1996) and PDW, which resembles aplastic anemia. The WBC count also decreased due to alcohol consumption. These conditions indicated to bone marrow dysfunction (Marietta et al., 1988). Silymarin and its liposomes improved these conditions moderately. This shows that prolonged treatment with silymarin and its liposomes may be beneficial in improving the condition.

The above findings were supported by increased bioavailability of silymarin. Extraction of drug from biological matrix is one of the crucial steps in pharmacokinetic study. Amongst the available methods of extraction, protein precipitation and liquid-liquid extraction (LLE) methods are the primitive methods and used widely in lab scale. In the present study, silymarin showed similar recovery from both methods. Thus, 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 biological matrix (like plasma) using protein precipitation method than LLE method (Prabu and Suriyaprakash, 2012). Another crucial step in bioanalytical method development involves selection of internal standard. Various compounds were tried namely, rutin, quercetin and α-naphthol. α-Napthol was found to be well resolved from the marker compound of silymarin (silybin(Pade, 2007)) and was earlier also used as internal standard by Wu et al., 2006 (Wu et al., 2006) for bioanalytical study of silymarin. The present bioanalytical method was developed by applying a few modifications in the analytical method proposed by Campodónico et al., 2001 (Campodonico et al., 2001) and Liu et al., 2007 (Liu et al., 2007) and further validated as per US-FDA guideline. Bioavailability study was performed in normal and 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 (Atul Bhattaram et al., 2002). Silybin A and silybin B showed similar pharmacokinetic profiles as reported by Yang et al., 2011 (Yang et al., 2011). The pharmacokinetic data were analysed by non-compartment modeling using WinNonlin software. The peak plasma concentration of silybin was achieved in 0.5 h in both alcoholic and normal rats. In normal rats, $C_{\text{max}}$ increased more than five times by conventional liposomes of silymarin compared to silymarin alone. This increase in $C_{\text{max}}$ was accompanied by a three-fold rise in AUC level and a decrease in half-life. The decrease in half-life can be justified by the fact that liposomes were cleared selectively by Kupffer cells. Alcohol-induced hepatotoxicity was developed in a similar way as for screening of drug and formulation. A similar pharmacokinetic profile was observed with a slight decrease in $C_{\text{max}}$ and AUC, which could be due to decrease in gastrointestinal absorption parameters of alcohol-intoxicated rat (Linnoila et al., 1979).