

Comparative Study of Different Silymarin Formulations: Formulation, Characterisation and *In Vitro/In Vivo* Evaluation

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Abstract: The aim of the present study was to study the synergistic hepatoprotective effect of silymarin with phospholipids when it is encaged in microspheres so as to passively target it to liver and to compare these silymarin formulations with silymarin solution. Various silymarin loaded lipid emulsions were formulated which include formulation A prepared with soyabean oil as an internal oily phase, soya lecithin as surfactant and tween 80 as cosurfactant; formulation B which was same as formulation A but was filtered through 0.45 μ membrane filter and finally steam sterilized for intravenous administration; formulation C containing soyabean oil as an internal oily phase, soya lecithin as surfactant, tween 80 and propylene glycol as cosurfactant/ cosolvent. These formulations were compared for their release profile with silymarin solution in propylene glycol, i.e. formulation D. *In vivo* evaluation was carried out using three models i.e. phenobarbitone induced sleep time in mice, biochemical estimation of SGOT and SGPT enzyme levels and histopathological examination of rat livers. Results revealed that there was significant reduction in sleep time in the mice treated with silymarin loaded lipid microspheres (both p.o. as well as i.v.) when compared with control and even with plain lipid microspheres and silymarin solution and significant reduction in enzyme levels in silymarin lipid microspheres treated group when compared with control, plain lipid microspheres as well as silymarin solution treated group. Histopathological studies also supported the results obtained from the other two models. A positive outcome of these studies gave an insight that if silymarin is coupled with phospholipid in such microparticulate delivery systems, hepatoprotective effect of drug molecules can be pronounced further by self targeting nature and synergistic action.

Keywords: Lipid microspheres, soya lecithin, silymarin, hepatoprotection.

1. INTRODUCTION

In pharmaceutical research, one of the major goals has been to synthesize or discover new chemical entities with desirable therapeutic properties but without undesirable effects. But the development of new drug molecule seldom fulfils the medical expectations and also searching new molecules demand heavily in terms of money, labour and time. And over and above all, the molecules often express their unwanted effect after a very long latent period, hence not time tested. Thus, in the light of these problems with the conventional research, scientists have always been in the search of changed strategies to improve upon the existing therapeutic armoury. Amongst many approaches, the one which has drawn a great deal of attention is "delivery-devices" wherein the therapeutic molecules are delivered at the site of action in a desired manner and in a right frame of environment, simulated by the carrier system. In this way, the same existing, old drugs can find a new potential in its intelligently designed delivery-vehicle and said to be given a new lease of life. The international scenario is quite encouraging in this newly emerged discipline better known as NDDS-novel drug delivery system. The drug delivery approaches, in general, aim to develop a carrier system which can hold the molecule effectively and then navigate them towards the right destination without affecting the

tissues en route and at the same time modifying and monitoring the drug-release character as well as drug-receptor interactions.

Various such systems, which have gained an utmost importance, include colloidal carriers like vesicular systems and microparticulate systems including liposomes, niosomes, pharmacosomes, microparticles, nanoparticles, [1] cellular carriers like resealed erythrocytes, antibodies, platelets and leukocytes [2]; supramolecular delivery systems like micelles, reverse micelles, mixed micelles, polymeric micelles, aquasomes, polymer based systems mucoadhesive, biodegradable-bioerodible polymers and soluble synthetic polymeric carriers [3]; macromolecular systems serum albumin, glycoproteins, antibody enzyme complex and biospecific antibodies, toxin and lectins [4].

Recently, the lipid emulsions which were, until now, have been popular for total parenteral nutrition (TPN), have drawn an attention, towards delivering the drugs, carried within membranous microstructures to the desired site and for effective interaction with the receptor present there [5-7]. Lipid emulsions are phospholipid based structures, also referred as lipid microspheres [6] and considered as a good potential substitute for much hyped closed bilayered, vesicular carriers (liposomes, niosomes, ufasomes etc.) as they resemble with them in many ways and could mimic their *in vivo* behaviour. Lipid emulsion preparation of corticosteroids, non-steroidal anti-inflammatory drugs and prostaglandins have shown more potent activity than free

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drugs. Colloidal nature and foreignness of lipid microspheres enable them to be dispatched to the liver as soon as they get into systemic circulation. Lipid emulsions mainly composed of phospholipids can themselves serve as hepatoprotectants. Essential phospholipids are known hepatoprotectives and also available in the market for the said purpose (eg. Essentiale™, Rhone Poulenc) [8]. Equipped with desired delivery potential to hepatic sites they have been considered here to strengthen the hepatoprotective action of silymarin. Silymarin, a group of flavonoid compounds obtained from *Silybum marianum*, is the chemically defined mixture of 3 isomers, silybin (major isomer), silychristin and silydianin. The drug is a unique hepatoprotective agent that has a positive effect on metabolism and physiology of liver cells, influencing their regenerative capacity due to two main actions: antioxidant and protein restoring activities. The drug also prevents toxic and foreign substances from penetrating liver cells by stabilizing the outer membranes of liver cells [9,10]. The present project is planned to explore the possibilities in the above-said direction by using lipids, lipidic surfactants and cosurfactants as components which can be designed or architected into lipid emulsions or lipid microspheres. The present study includes the incorporation of silymarin into various lipid microsphere formulations, their *in vitro* characterisation and *in vivo* evaluation.

2. MATERIALS AND METHODS

Silymarin was gift from Ranbaxy Laboratories Ltd., Soyabean lecithin was generous sample from Natterman Phospholipids (Germany), tween 80, and propylene glycol were purchased from S.D. Fine Chemicals, Mumbai (India). Soyabean oil, was obtained from Protina (India). All other materials used in the study were of analytical grade.

2.1. Preparation of Lipid Emulsion

Soyabean lecithin was dispersed well in soyabean oil and allowed to dissolve completely by keeping it overnight at 50°C. The cosurfactants (hydrophilic/lipophilic) were added to the oily or aqueous phase as per their solubility. The aqueous phase was added to the oily phase dropwise with simple agitation to obtain lipid emulsion. Silymarin solution (10% w/v in 1M sodium hydroxide) was added to the prepared emulsion so as to obtain the final concentration of 10 mg/g at a temperature of 50°C under stirring with magnetic stirrer. After 10-15 minutes, the pH was adjusted to 7.4 with 1N orthophosphoric acid [11].

2.2. Structure and Morphology

Morphology and structure of lipid microspheres was determined using Transmission electron microscopy (TEM) and photomicrographs were taken at suitable magnifications (Fig. 1).

2.3. Size and Size Distribution Measurements

Size of the globules was determined with the help of Master sizer (Malvern instruments Ltd., UK) using Laser light scattering technique.

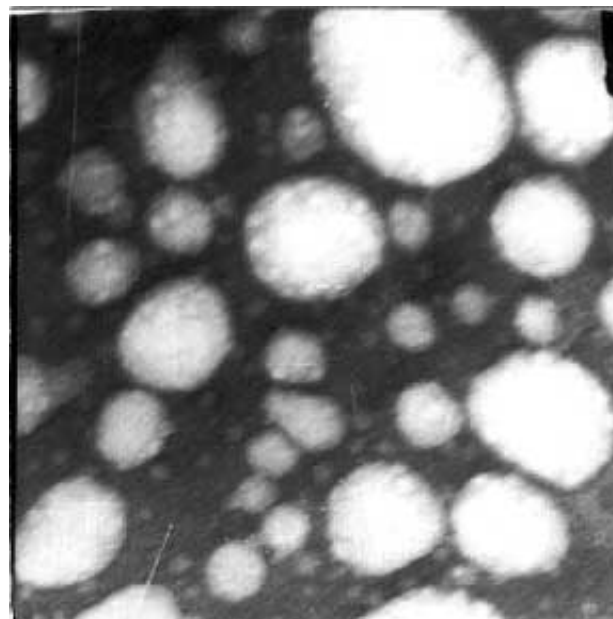


Fig. (1). Transmission electron photomicrograph of silymarin loaded lipid microspheres.

2.4. *In Vitro* Release Rate Studies

These studies were carried out through dialysis bag using dissolution apparatus USP I. The treated dialysis membrane was soaked in diffusion medium (Phosphate buffer pH= 7.4) upto 48 hours and refrigerated till use. The membrane was washed with distilled water before use. The formulations A, B, C and D (Table 1) each equivalent to 20 mg of silymarin were filled in dialysis bags and placed in basket of dissolution apparatus USP I. The Study was run at 50 rpm and dissolution vessel was maintained at 37±0.5°C. An aliquot of 5 ml of samples was withdrawn at suitable time intervals till 36 hours and replaced with same amount of medium to maintain the volume of dissolution medium as 900 ml. The samples were quantitated by UV spectrophotometer at 287 nm (Fig. 2).

2.5. *In Vivo* Pharmacological Evaluation

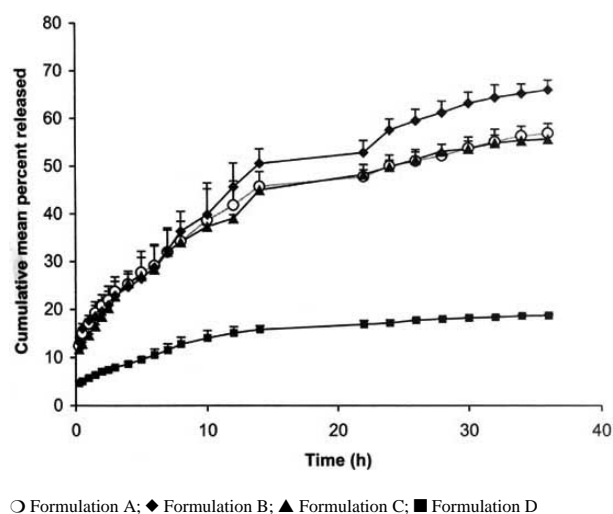
In vivo evaluation of the prepared silymarin lipid emulsions was performed using following models. Animals were housed in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC). Temperature was maintained at 22°C and daily temperature fluctuations were kept to a minimum (± 2°F) to avoid large demands on the animal's metabolic and behaviour processes. Relative humidity was 60% (± 10%). Air exchange rate of 12 (± 2) changes per hour was maintained. Animals were maintained on standard pellet food and water ad libitum with 12h light dark cycle periods (8.00am to 8.00pm)

Model 1: Phenobarbitone Induced Sleep Time in Mice

The method reported by Recknagel [12], was used for hepatoprotective activity. Carbon tetrachloride (CCl₄), a well known hepatotoxin was used in dose of 0.15 ml/kg mixed with liquid paraffin and was administered orally. The

Table 1. Various Silymarin (1%) Formulations

Components	FORMULATIONS			
	A	B	C	D
Silymarin (10% solution in 1M NaOH)	1%	1%	1%	1%
Soyabean oil	5%	5%	5%	×
Soya lecithin	1.2%	1.2%	1.2%	×
Tween – 80	4%	4%	4%	×
Propylene glycol	×	×	10%	q.s
Water	q.s	q.s	q.s	×
Filtered through 0.45 μ nylon filter and steam sterilized	×	✓	×	×



○ Formulation A; ◆ Formulation B; ▲ Formulation C; ■ Formulation D

Fig. (2). Comparison of cumulative mean percent released vs time (h) for various formulation (A-D).

animals (male laca mice, 25-30g body weight) were divided into eight groups (each group consists five animals). The various silymarin contained lipid emulsions were freshly prepared and administered to animals in group through the routes indicated in Table 2. Formulation A contained silymarin lipid emulsion for oral administration, formulation B for i.v. administration (IV injection was administered through caudal vein of mice in awoken animal. Volume administered was adjusted according to weight of mice i.e. 0.1 ml/10g of body weight), formulation PLE (plain lipid emulsion without silymarin) and formulation D contained silymarin in solution for oral as well as i.v. administration. The treatment schedule and effects were recorded. (Table 2).

Sleep time was determined for various groups & hepatoprotective activity was calculated by the following formula [13].

$$1 - \frac{T_d - T_n}{T_c - T_n} \times 100$$

where T is the sleep time, c, n, d are control normal and drug treated group of animals, respectively.

Results were interpreted using one way ANOVA followed by Dunnett's test.

Model 2: Estimation of SGOT and SGPT Enzyme Levels in Rats

The animals (Wistar rats, 100-150g, were divided into four groups (n=5 in each group) (Table 3)

Basal SGOT/SGPT Enzyme Levels

The blood was withdrawn from the tail vein and serum was separated from it by centrifugation for the estimation of SGOT and SGPT enzyme levels using method of Reitman and Frankel.

GROUP 1 :Control

Hepatotoxin, CCl₄ (1ml/kg, p.o.) was administered to each animal in the group. After a lapse of 2 hours, serum was isolated for the estimation of SGOT and SGPT levels.

GROUP 2: Silymarin Lipid Emulsion (SLE)

Suitable volume of SLE (equivalent to 50 mg/kg of silymarin) was administered orally followed by CCl₄ (1ml/kg, p.o.) after one hour interval. The levels of SGOT and SGPT were determined in the serum isolated after 2h and 24h of the CCl₄ treatment.

GROUP 3: Plain Lipid Emulsion (PLE)

Plain lipid emulsion (volume equivalent to silymarin lipid emulsion) was administered orally 1h prior to the CCl₄ (1 ml/kg, p.o.) administration. Serum was isolated after 2h and 24h of CCl₄ administration to estimate SGOT & SGPT levels.

GROUP 4 : Silymarin Solution

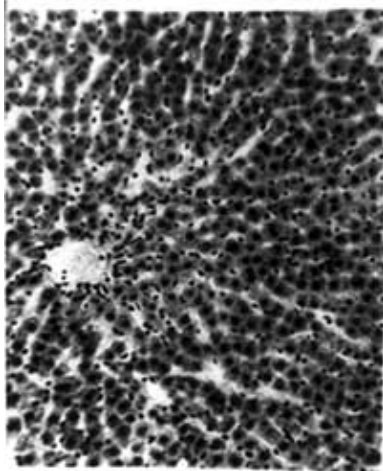
Silymarin solution (equivalent to silymarin 50 mg/kg, p.o.) was administered 1h prior to CCl₄ (1ml/kg, p.o.) administration. SGOT and SGPT levels were measured in the serum isolated after 2h and 24h of CCl₄ administration.

Model 3 : Histopathological Examination

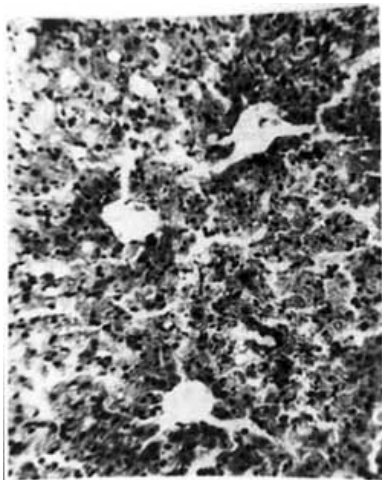
After 24h (in the above experiment i.e., Model 2), Livers of the experimental animals were fixed in 10% formosaline (10% v/v formaldehyde in normal saline) for 48h and processed for paraffin embedding following the standard microtechniques. 5 μ thick sections of the paraffin-embedded livers were cut using a rotary microtome. Alum-haematoxylin and eosin were used for staining the liver sections. Deparaffinised slides were downgraded through various grades of ethanol to water. The sections were then stained in alum-haematoxylin for 1h, rinsed in water and differentiated in acid water till the nuclei turned pink. At this stage, the slides were dipped in the ammonia water to make the nuclei blue. The slides were then upgraded till 90% ethanol, counterstained with eosin (2-4 minutes), differentiated in 90% ethanol, dehydrated in absolute ethanol and cleared in xylene before mounting. The stained sections were examined under the light microscope and photographs of the sections were taken with Asahi Pentax Spomatic F 35mm camera fitted with Super-Multi coated 1:4/50 Macro-Takumar lens.

Photomicrographs (Fig. 3a, b, c, d and e) of the liver sections of rats in different groups were also taken.

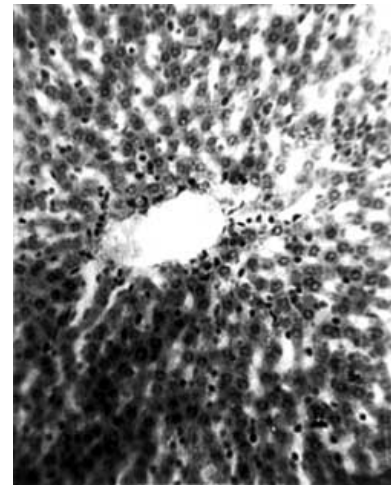
a)



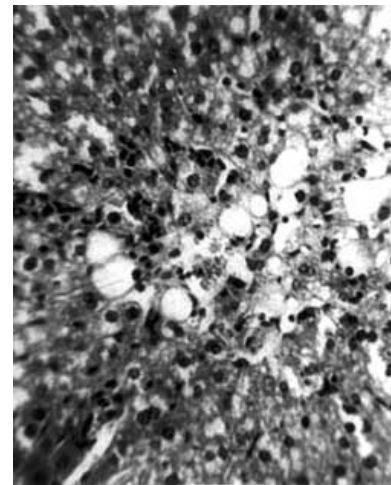
b)



c)



d)



e)

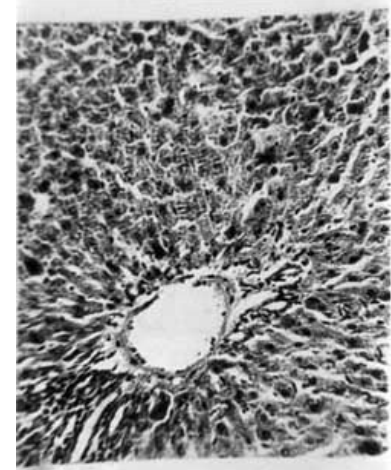


Fig. (3). a. Histopathology of liver in normal animals
 b. Histopathology of liver in carbon tetrachloride treated animals
 c. Histopathology of liver in animals treated with silymarin lipid emulsion (SLE) (after carbon tetrachloride treatment).
 d. Histopathology of liver in animals treated with plain lipid emulsion (PLE) (after carbon tetrachloride treatment).
 e. Histopathology of liver in animals treated with silymarin solution (after carbon tetrachloride treatment).

3. RESULTS AND DISCUSSION

3.1. Shape, Size & Size Distribution Measurements

Results of transmission electron microscopy (as can be seen from photomicrograph in Fig. 1) revealed that the lipid emulsion microdroplets were almost spherical in shape having appearance of adsorbed or coated surface. Coated surface can be identified as flexible interfacial film formed with the emulsifier and co-emulsifier. The size distribution range was from 0.31–1.24 μm with the most frequent size of 0.632–0.732 μm . The median diameter (the diameter for which 50% of the particles measured were less than that size) was found to be 0.46 μm . 10% of the measured particles were less than 0.33 μm while 90% of the measured particles were less than 0.89 μm . The specific surface was found to be 10.7592 sq. m/g.

The submicron range and a good uniformity in size may be ascribed to the total composition of the prepared lipid emulsion in which the optimum level of cosurfactant (Tween 80) intercalates with that of surfactant (lecithin) to provide microdroplets of appropriately chosen oily core the interfacial barrier of desired strength and efficiency.

3.2. In vitro Release Rate Studies

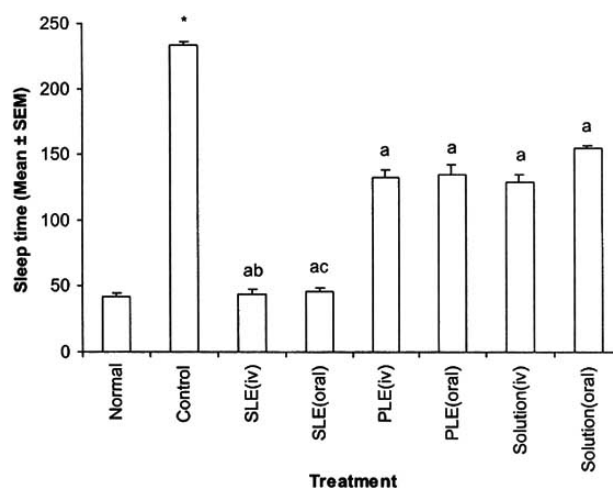
The prepared silymarin lipid emulsion (Formulations A to C) were compared with the silymarin solution (Formulation D) with respect to their drug release behaviour. The formulations A to C have shown almost similar release rate profile with mean percent release of 56.70 ± 2.039 for formulation A, 65.72 ± 0.783 for formulation B and 55.48 ± 1.831 for formulation C after 36 hours which was only 18.67 ± 0.192 for the silymarin solution (Formulation D) (Fig. 2). The results revealed that the addition of propylene glycol to the prepared silymarin lipid emulsion did not affect its release behaviour and thus there was no further advantage of its addition as cosurfactant to the formulation. Also the filtration and autoclaving did not affect the drug release profile of the formulation that suggested that the similar drug release characteristics can be achieved even after membrane filtration and autoclaving the lipid emulsion. The faster and the better drug release from the silymarin lipid emulsion as compared with silymarin solution may be due to the fact that the surficial presence of the surfactants in the prepared silymarin lipid emulsions help to carry the drug in the solubilized form towards the outer domain of oily sphere thus can better transport it from the surface of the system through the dialysis membrane. While the silymarin in the solution form tends to be retained there in the solution itself due to poor diffusion through the dialysis membrane, viscosity of the solution due to propylene glycol might have rendered the drug poorly diffusible through dialysis membrane and hence only a maximum of 18.67 ± 0.192 mean percent drug was released in 36 hours.

This suggested that the submicron range silymarin lipid emulsion can help diffuse the drug in a pronounced manner to increase the bioavailability of the drug so that, it can reach the system in required concentration.

3.3. In Vivo Pharmacological Evaluations

The hepatoprotective action of silymarin on the duration of sleep induced in CCl_4 treated mice by phenobarbitone

sodium indicated the improved performance in silymarin lipid emulsion (SLE) at a level of 98.9% on intravenous administration and 97.9% on oral administration. The plain lipid emulsion (PLE) also showed hepatoprotection of 52.8% on intravenous administration and 51.8% on oral administration while the silymarin solution provided the hepatoprotection of 54.9% on intravenous administration and of 41.36% on oral administration (Table 2 and Fig. 4, 5). The hepatoprotective activity of silymarin loaded lipid emulsion was statistically significant (at 95% confidence level on one way ANOVA followed by Dunnett test) than that of the plain lipid emulsion and drug solution. With regards to the route, the studies revealed that there has been almost an equal of hepatoprotection after oral and i.v. administration (Fig. 4,5). The oral efficiency of lipid emulsion can be accounted to the submicron range phospholipid coated chylomicron like



* $P < 0.05$ as compared to normal; ^a $P < 0.05$ as compared to control; ^b $P < 0.05$ as compared to PLE iv and solution iv; ^c $P < 0.05$ as compared to PLE oral and solution oral

Fig. (4). Comparison of sleep time for various treatments in mice.

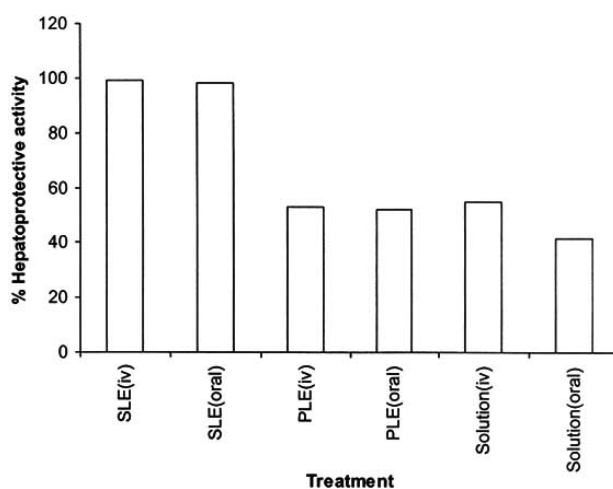
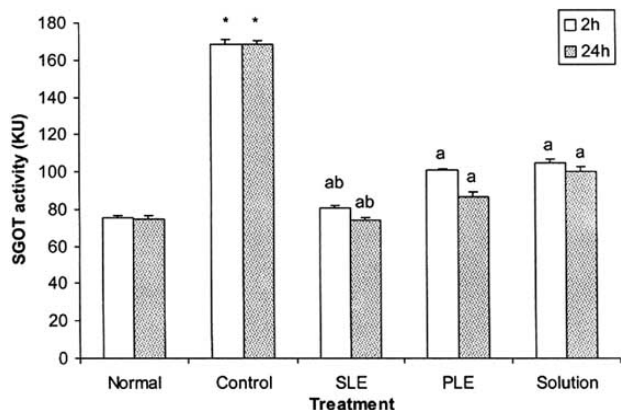


Fig. (5). Percent hepatoprotective activity in mice with various treatments.

structures. The later find easy way through the endothelial barrier of the gastrointestinal tract and then enter the blood circulation very quickly where they are quickly dispatched to the hepatic parenchymal cells [14]. The selective disposition of microemulsified lipid carriers could be due to its small size range (0.36-1.24 μm) which otherwise may have trapped in the RES of liver [15,16]. Further, phospholipid coated lipidic microstructures may have found better target cell (hepatocyte) interaction (as in case of liposomes) to merchandise therapeutic molecules more effectively.

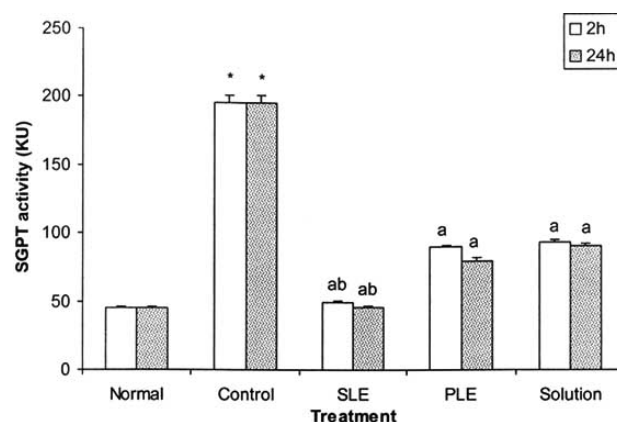
The biochemical parameters i.e. SGOT and SGPT enzyme activity (expressed in Karmen Units KU) were also determined in each group. SGOT and SGPT levels were found to be raised to 168.02 ± 2.38 and 196.2 ± 5.22 from the initial value of 75.04 ± 1.55 and 45.176 ± 1.25 respectively after CCl_4 treatment in control group. On administration of silymarin contained lipid emulsion there was noted to be a significant lowering of SGOT and SGPT (Fig. 6 and 7) enzyme activity after 2 hrs and after 24 hrs of the CCl_4 administration in all the groups. Also the lowering of SGOT and SGPT enzyme activity with silymarin lipid emulsion was observed to be statistically significant than that with plain silymarin solution and plain lipid emulsion. The enzyme level changes have in consonance to sleep time observations and substantiate the efficacy of lipid emulsion of silymarin. The improvement in the enzyme activity was due to the membrane stabilization effect of both the phospholipids as well as silymarin on the hepatocytes which may have prevented the penetration of the hepatotoxin (CCl_4) and hence the leakage of SGOT and SGPT enzymes from the liver cells.



* $P < 0.05$ as compared to normal; ^a $P < 0.05$ as compared to control; ^b $P < 0.05$ as compared to PLE and solution

Fig. (6). Effect of various treatments on the SGOT activity after 2h and 24h of the CCl_4 administration.

In the histopathological studies, a picture of normal hepatocytes of liver can be seen in photomicrograph 3a while in photomicrograph 3b, the enormous damage of liver could be seen due to the CCl_4 intoxication. But in photomicrograph 3c, almost complete protection of the liver cells from CCl_4 damage could be seen when silymarin in lipid emulsion was given orally. With plain lipid emulsion, a partial protection



* $P < 0.05$ as compared to normal; ^a $P < 0.05$ as compared to control; ^b $P < 0.05$ as compared to PLE and solution

Fig. (7). Effect of various treatments on the SGPT activity after 2h and 24h of the CCl_4 administration.

of hepatocytes with hepatotoxin was seen as depicted in photomicrograph 3d. Silymarin in solution had also shown a very little protection as compared to SLE that might be due to its incomplete or lesser bioavailability to the liver cells, when present in solution as shown in photomicrograph 3e.

The improved performance of silymarin may be well assigned to the triglycerides and phospholipid. These are known to be taken up passively by the liver and thus could carry the drug molecules along with to the hepatic site. Thus they serve as vector for silymarin molecules to target them passively at the hepatic site. Also the silymarin possess an ability to form complex with phosphatidylcholine [17-19], which provides better stability in the biological milieu and thus lesser loss of drug en route.

Further, the enhanced activity may be ascribed to the synergistic or dual effects of phospholipids and drug per se with regard to

- i) Stabilization of hepatocyte membrane to prevent the penetration of toxicants by altering phospholipid turnover.
- ii) Inhibitory effect on lipid peroxidation as the two phospholipids and drug are known antioxidants. The later known to be 10 times more potent as antioxidant than -tocopherol.

4. CONCLUSION

The potential of silymarin in lipid microspheres was found to be enhanced when tested on animal models. This proves that carriers like lipid microspheres can help to navigate and negotiate the silymarin molecules *in vivo* in an effective way for hepatoprotection. Remarkably, oral administration of silymarin loaded lipid microspheres showed performance at par to that of i.v. administration. Oral efficacy of silymarin in lipid microspheres unfolds its potential through this convenient route which holds further future promises for other agents as well which are to be targeted to the hepatic site.

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