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PARENTERAL SUSTAINED RELEASE SILYBIN MICROPARTICLES INTENDED FOR TREATMENT OF LIVER FIBROSIS

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ABSTRACT

The objective of this work was to prepare biodegradable sustained release parenteral microparticles of silybin, characterize the formulation and evaluate the hepatoprotective activity of the microparticle formulation. Silybin microparticles were formulated by o/w emulsion solvent evaporation technique using poly-ε-caprolactone as polymer. Four different microparticle formulations (MP1, MP2, MP3 and MP4) were prepared by varying the drug/polymer ratio. The particles were characterized for particle size, drug content, surface morphology and in vitro drug release. The hepatoprotective activity of the formulation was determined in a CCl_4-treated rat model and also compared with silybin solution. The mean particle size of prepared silybin microparticles was found to be 7.5-25µm, spherical shape with smooth surface. The encapsulation efficiency was found to 89-94%. The in vitro release of silybin was relatively fast initially followed by a slower and sustained release. From the four formulations MP1 was selected as best formulation by considering the size, entrapment efficiency and release profile. Silybin microparticular administration reversed serum enzyme levels. The developed silybin microparticles showed superior hepatoprotective activity to silybin solution.

KEY WORDS

Silybin, Microparticles, Biodegradable polymer, Pharmacodynamics, Hepatoprotective activity.

INTRODUCTION

The liver, which is the major organ responsible for the metabolism of drugs, toxic chemicals and byproducts endogenous to the body, is also the primary target organ for detoxication of many endogenous and exogenous toxic chemicals[1-2]. The proportion of major liver diseases, such as non-alcoholic and alcoholic fatty liver, chronic hepatitis, fibrosis, cirrhosis or hepatic carcinoma leads to a severe death causing diseases in both human beings and animals [3]. Hepatic fibrosis is the accumulation of extracellular matrix, or scar, in response to acute or chronic liver injury. Fibrogenesis represents a wound healing response to injury, and ultimately leads to cirrhosis. Cirrhosis, the end state of the fibrotic process, is characterized by dramatic accumulation of ECM in the liver resulting in nodule formation [4]. There is no standard treatment for liver fibrosis [5]. Thus there is a need to find effective treatment for fibrosis.

The ideal antifibrotic therapy would be one that is liver-specific, well tolerated when administered for prolonged periods of time, effective in attenuating excessive collagen deposition without affecting normal ECM synthesis, effectively delivered and nontoxic to other organs[6]. Silybin is one of the oldest drug

which is used for the treatment of liver disorders. Silybin is mainly extracted from the seeds of the plant "SilybumMarianum", which is commonly known as Milk thistle [7-9]. Silybin also known as Silybinin is a mixture of two diastereomers A and B in approximately 1:1 proportion. Silybin is a hepatoprotective agent useful in the treatment of fibrosis and cirrhosis, both of which are chronic diseases and require long term drug therapy. The major problems with many of the hepatoprotective drugs are they are neither liver specific nor fibrosis specific. As a result insufficient amount of the drug may accumulate in the liver (target cell). Moreover uptake of the drug innon-target cells is the principal cause of serious adverse effects [10]. In liver diseases silybin drug is chronically administered orally every day. The effectiveness of oral silybin as a hepatoprotective agent was discounted by its poor solubility and low bioavailability due to high first pass metabolism and low $t_{1/2}$ [8]. To overcome this problem sustained release microparticulate delivery system was utilized for encapsulation of silybin. Particulate carriers usually accumulate in the liver by passive targeting upon parenteral administration. Sustained release drug delivery systems are used to improve the therapeutic response by demonstrating blood levels that are more consistent and stable compared to immediate release dosage forms. They can result in higher tissue levels compared to conventional dosage forms as there is a continuous supply of drugs into the tissue with no troughs. In this study, we compared the hepatoprotective activity of silybin using a sustained release formulation developed in this study with an equivalent parenteral I.V. formulation. Thus, the objective of this study was to prepare a sustained release parenteral depot for silybin, a well-known hepatoprotective agent and further to evaluate itsin vivo performance.

MATERIALS AND METHODS

MATERIALS

Silybin and Poly-ε-caprolactone (molwt, 14,000) were procured from Sigma-Aldrich, Germany. Polyvinyl alcohol (PVA, cold-water soluble) was procured from Qualikems Fine Chemicals Pvt Ltd, New Delhi. Dichloromethane was procured from SD Fine Chemicals Ltd, Mumbai, India. All other reagents were of analytical grade. HPLC (Waters, USA) was used to analyze plasma samples while UV-Visible spectrophotometer (Shimadzu UV-1800, Japan) was used to analyze drug loading and drug release samples. A magnetic stirrer (RemiIndustries, Mumbai, India) was used to facilitate evaporation of dichloromethane whilean ultracentrifuge (Remi, Mumbai) was employed to recover the nanoparticles after preparation. Male Wistar rats weighing 150 – 180g were purchased from MahaveerEnterprises, Hyderabad., India.

METHODS

Preparation of silybinmicroparticles

Emulsion (O/W) solvent evaporation method was employed in the preparation of silybinmicroparticles using poly-ε-caprolactone as the polymer. Four different microparticle formulations MP_1, MP_2, MP_3 and MP_4 containing drug: polymer in the ratio of 1:1, 1:2, 1:3 and 1:4, respectively, were prepared. For the preparation, silybin (100mg) and polycaprolactone (100 mg) was dissolved in 15 ml of dichloromethane by vortexing. The mixture(organic phase) was added drop-wise to 50ml of 2% PVA solution whilestirring by use of magnetic stirrer to obtain a o/w emulsion. Stirring was continued until the organic solvent was completely evaporated; at the end microparticles were formed.After evaporation of

organic phase the solution was filtered and dried to collect micro particles.

In Vitro characterization of Microparticles:

The microparticle formulations were evaluated for particle size, drug-excipient interaction, and in vitro drug release. Percentage yield was calculated.

$$\text{Percentage yield} = \frac{\text{Practical weight}}{\text{Theoretical weight}} \times 100$$

Determination of particle size:

The mean particle size of the silybin microspheres were determined by optical microscopy. The eyepiece micrometer was calibrated using a stage micrometer and the calibration is undertaken to find out the measure of each division using the stage micrometer. After calibration, the eye piece micrometer is used for determining the size of microspheres. The microspheres was mounted on a slide and observed under the microscope. At least 200 micro spheres were measured for each preparation and the mean diameter was calculated. The shape of the microspheres was visualized and the photographs were taken with the aid of binocular microscope (QUASMO, ANISO, PZRM 700 India 9001-2000).

Determination of encapsulation efficiency:

An accurately weighed 20 mg microspheres were dissolved in 10ml of dichloromethane. The absorbance associated with the dissolved silybin was determined. A 20 mg of blank microspheres were also dissolved in the same solvent and absorbance measured at the same wavelength. The absorbance associated with the silybin was a substraction of the absorbance of the silybin microspheres and the blank microspheres. Drug content was calculated from the standard curve. Percentage Encapsulation Efficiency (EE) was calculated by using the formula:

$$\text{Encapsulation Efficiency} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100$$

Particle shape and surface morphology

Morphological characterization of the microparticles was done by using Scanning electron microscope (JEOL JSM -5200). The samples were coated to 200A° thickness with gold-palladium using prior to microscopy. Microcapsules before dissolution study were only subjected to SEM study.

In vitro release study

The in vitro release study was performed in a diffusion cell developed in house. An inverted cylindrical test tube cut to a height of 8cm was used as a donor cell. The receptor compartment consisted of 100 ml of phosphate buffer (pH 7.4). A dialysis membrane soaked in warm water for 30 minutes was placed at the lower end of the cylindrical portion. Microparticles containing 20mg of drug was suspended into 5ml of pH 7.4 buffer and placed in the donor compartment. The donor compartment was inserted into the receptor compartment such that the height was sufficient for the drug to be released into the receptor. The system was stirred using a magnetic stirrer and bead.Samples (5ml) were removed from the receptor compartment and replaced with the same volume of fresh medium immediately. The samples were analyzed spectrophotometrically at 287nm.

In vivo studies

Male Wister rats (weighing 150–180g each) were purchased from MahaveerEnterprises, Hyderabad, India, and were maintained in an air-conditioned room at 22 ± 2 ^{0}C and relative humidity of 45–55% in a 12/12 h light/dark cycle. The animals had free access to standard food pellets and water was available ad libitum. All the animal experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of

Experiments on Animals (CPCSEA), Chennai, India [11] and the study protocol was approved by Institutional Animal Ethical Committee of Vaagdevi College of Pharmacy, Warangal, India (ref no. 1047/ac/07/CPCSEA). International guidelines issued by the International Council for Laboratory Animal Science were also followed [12]. These conditions were maintained throughout the duration of the experiment.

EVALUATION OF HEPATOPROTECTIVE ACTIVITY

Carbon tetrachloride (CCl_4)-induced liver damage model was used in the evaluation of hepatoprotectiveactivity [13]. For this purpose another set of male Wistar rats were divided into five groups each containing 6 rats.Group1 received normal saline (1ml/rat) daily for 9 days and served as normal control. Group 2 received CCl_4 (dissolved in 3 times its volume of olive) at a dose of 0.7 ml/kg intraperitoneally on days 3, 6 and 9 and served as toxic control. Group 3 received the drug solution in a dose of 100 mg/kg intraperitoneally daily for 9 days. Group 4 received silybinmicroparticular suspension equivalent to 100 mg/kg of drug intraperitoneally on day 1 while Group5 received placebo microparticles. All the groups received CCl_4 at days 1, 3, 6 and 9 of the study except normal control.

The animals were anaesthetized on the last day of the study and blood was collected by cardiac puncture. Plasma was separated from the blood samples by centrifugation at 3000rpm for 15min. Hepatoprotective activity was quantified by the Serum glutamate oxaloacetate transaminase (SGOT) and Serum glutamate pyruvic transaminase (SGPT) levels present in the plasma. Subsequently, their livers were subjected to histopathologicalexamination. First, the rats were sacrificed at the last day of the study,the liver separated carefully and preserved in formalin solution, and liver sections were prepared. The body weights of the rats were also monitored.

Statistical analysis

The data was expressed as mean± standard deviation (SD) and statistical analysis was carried out by one-way ANOVA followed by Student's Newman-Keuls test. The level of significance used was $P < 0.05$. The statistical software used was Graph Pad Prism, USA, versions 4 and 5.

RESULTS AND DISCUSSIONS

Silybinmicro particles were successfully prepared using poly-ε-caprolactone by o/w emulsion solvent evaporation method. The mean particle size of the micro particles ranged from 7.5 - 25 µm (**Table 1**). Mean particle size increased with increase in polymer concentration. The encapsulation efficiency of the micro particlesincreased as polymer concentration increased. The SEM micrographs of silybin micro particles samples, shown in **Figure 1** revealed that the microparticles were smooth and with spherical in shape.

In vitro release profiles of micro particles were shown in the **Figure 2**. The release study was performed for 10 days. Samples were withdrawn at interval of 24 hours up to 10 days and absorbances were measured by UV spectrophotometer at 287nm. A biphasic drug release pattern was found, i.e., burst release followed by sustained release.In the first 6 hours of release, the unencapsulateddrug component was released. Subsequently, the encapsulated drug component was gradually released. Drug release from MP1 was higher than from MP2, MP3 and MP4.The percent cumulative drug release was observed to be 95, 89, 85 and 80 and in vitro drug release sustained up to 10 days for MP_1, MP_2, MP_3 and MP_4, respectively. The release profiles were fitted into various release kinetic models the r^2 values were shown in **Table**

2. From the release data we observed that increase in the polymer content delays the drug release due to increase particle size and reduced surface area available for drug release.

The Silybin formulations were screened for hepatoprotective activity in rats. The administration of CCl_4 to the animals resulted in a marked increase in SGPT and SGOT levels. This toxic effect was controlled in the animals treated with Silybin formulations. Both biochemical and histopathological changes were observed for the assessment of liver function. Histological profile of the control animals showed normal hepatic architecture with distinct hepatic cells, well presented cytoplasm sinusoidal spaces and central vein (**Figure 4a**). Disarrangement of normal cells with intense centrilobular necrosis was observed in CCl_4 intoxication liver (**Figure 4b**). Moderate accumulation of fatty lobules and cellular necrosis were observed in the animals treated with Silybin solution (**Figure 4c**) and micro particular formulation exhibited a significant liver protection against CCl_4 induced liver damage, as evidenced by the presence of normal hepatic cords and well defined cytoplasm and absence of necrosis (**Figure 4d**). The effect of silybin formulations on enzyme levels in rats with carbon tetrachloride (CCl_4) -induced hepatotoxicity were shown in **Table 3**.

Table 1: Particle size and encapsulation efficiencies of Silybinmicroparticles

Formulation	Particle size (µm)	%Encapsulation efficiency
MP_1	10 ± 2.5	89
MP_2	12 ± 3.4	91
MP_3	15 ± 3.2	92.3
MP_4	21 ± 4.5	93.1

Table 2: Release Kinetic models and their r^2 values of SilybinMicroparticles

Formulation code	Zero order	First order	Higuchi	Peppas	
	r^2	r^2	r^2	r^2	n
MP1	0.926	0.9839	0.9929	0.9837	0.437
MP2	0.9299	0.9895	0.9932	0.9688	0.48
MP3	0.9394	0.9883	0.9946	0.9575	0.526
MP4	0.9484	0.988	0.9916	0.9532	0.555

Table 3: Effect of silybin formulations on enzyme levels in rats with carbon tetrachloride (CCl_4) - induced hepatotoxicity (mean ± SD, n = 6)

Treatment Group	Initial body weight (g)	Body weight after 9 days (g)	SGPT(U/L)	SGOT(U/L)
Control	160±10	180±15	12.1±2.3	33.3±2.9
CCl_4	165±15	148±10	77.8±3.7	90.0±3.9
Silybin solution	170±5	180±5***	44.4±4.6***	63.3±2.8***
Silybinmicroparticles	162±7	182±12***	19.4±3.2***	43.6±4.8***
Placebo microparticles	165±6	156±12	70.4±2.3***	86.5±2.5

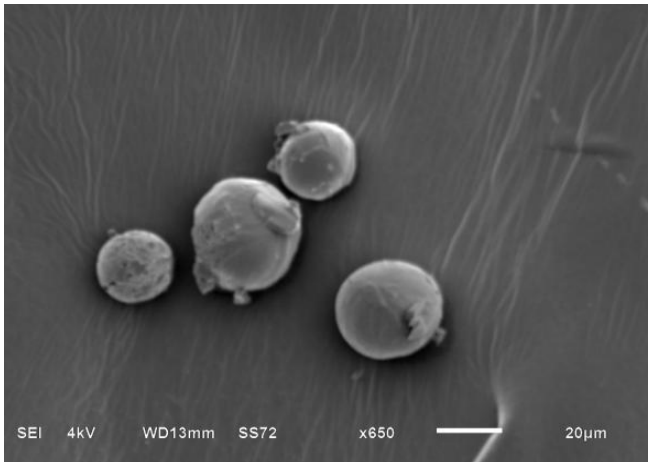


Figure 1: Scanning electron microscopic (SEM) Image of Silybinmicroparticles.

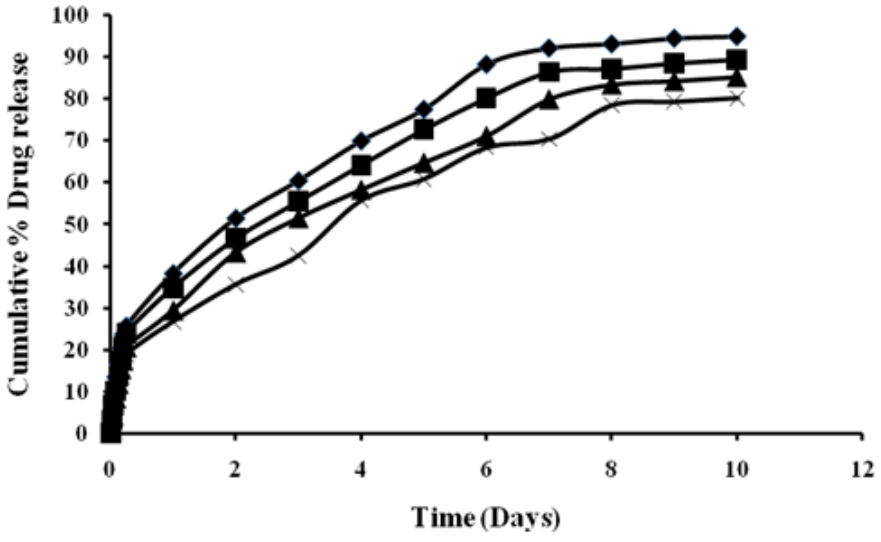


Figure 2: Drug release fromsilybinmicroparticles (◊= MP1, □=MP2, Δ= MP3, × =MP4)

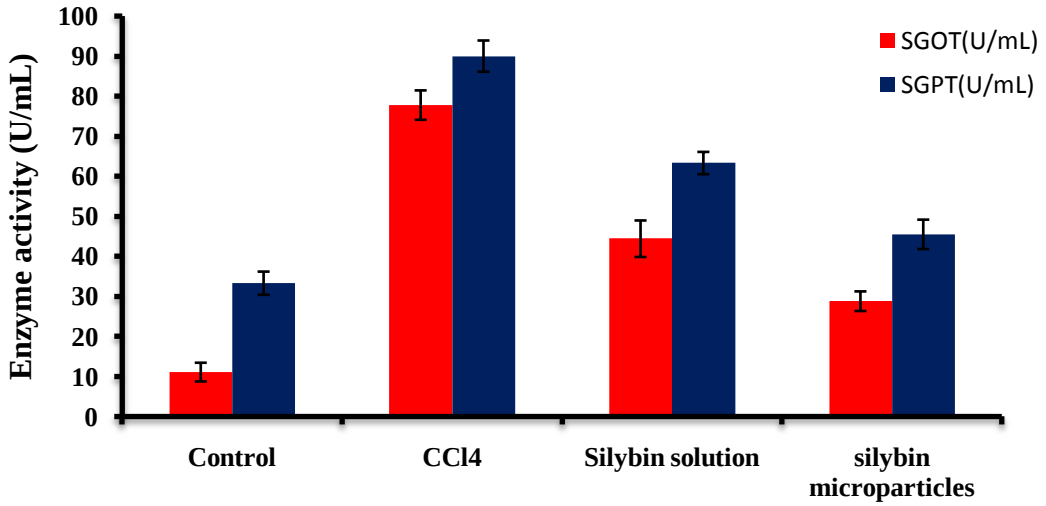
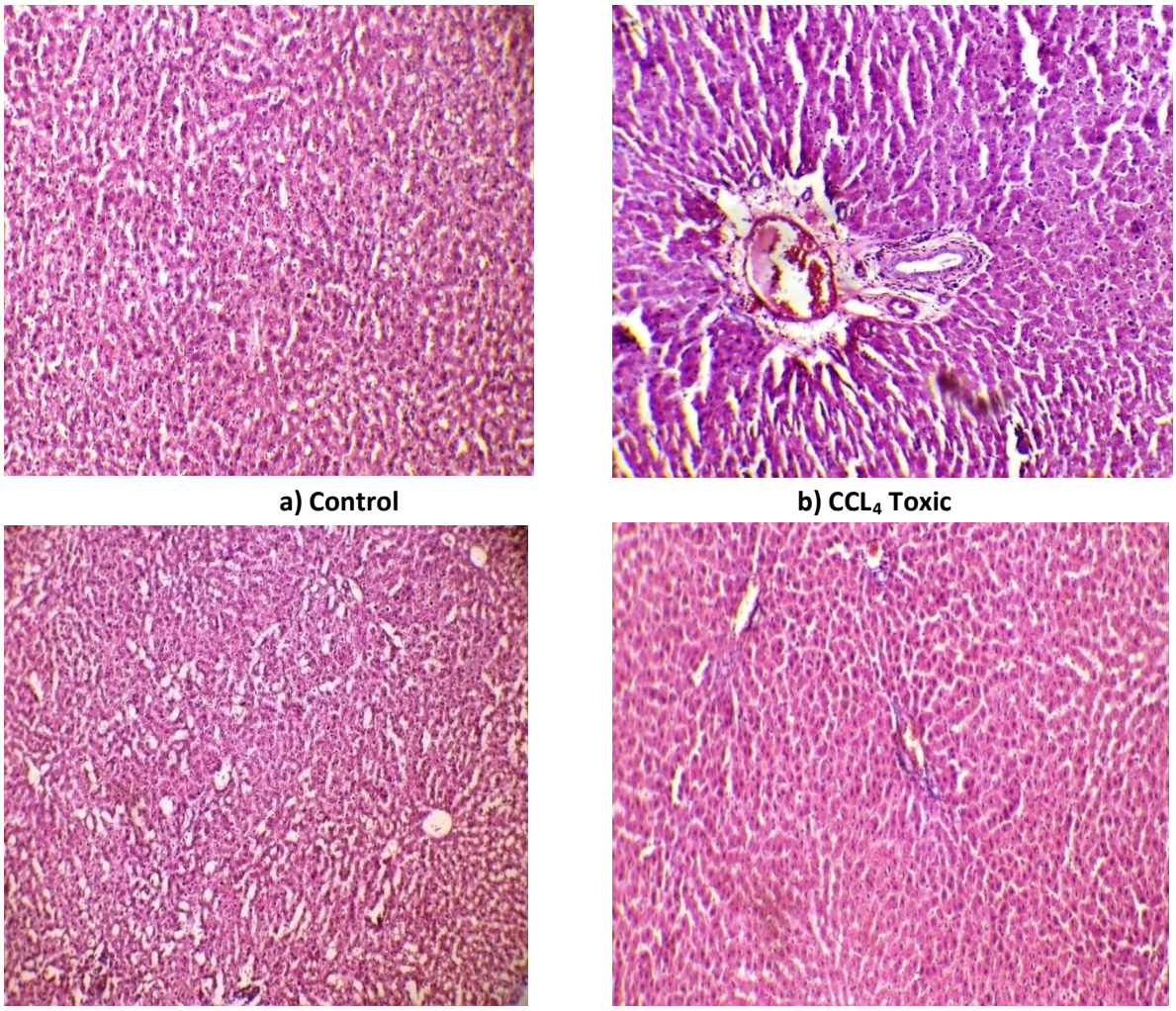


Figure3: Effect of silybin and its formulations on Liver Enzyme Levels in rats with CCl_4 induced hepatotoxicity.



a) Control **b) CCL_4 Toxic**

c) Silybin solution **d) Silybinmicroparticles**

Figure 4: Histopathology of rat livers

To achieve the aim of this study, silybinmicroparticles have been prepared using emulsion solvent evaporation technique. The technique of emulsion solvent evaporation offers several advantages. It is preferred to other preparation methods such as spray-drying, sonication and homogenization, etc., because it requires only mild conditions such as ambient temperature and constant stirring. We used polycaprolactone, a biodegradable polymer, to prepare microparticles. Polycaprolactone is a biocompatible, bioabsorbable, and biodegradable aliphatic polyester polymers that degrades slowly and does not generate an acid environment unlike the polylactide (PLA) or polyglycolide (PLG) polymers. Although the permeability of macromolecules in PCL is low, such low permeability may be sufficient for drug delivery [14]. Other advantages of PCL include hydrophobicity, in vitro stability and low cost.

Particle size and entrapment efficiency of both silybinmicroparticles were increased with increasing the polymer content. This may be due to availability of more polymer to coat the drug. From the release data we observed that increase in the polymer content delays the drug release due to increase in particle size and reduced surface area available for drug release. From all the formulations MP1 was used for determining hepatoprotective activity as optimized formulation because of less particle size and good release profile.

To test the hepatoprotective activity of microparticles, the formulation was administered to CCl_4 induced model. Carbon tetrachloride, a known hepatotoxin is a commonly used model for hepatoprotective drug screening, and the severity of the liver damage is measured by the levels of elevated cytoplasmic enzymes (SGOT and SGPT) in circulation [15]. The hepatocellular damage induced by CCl_4 is due to its metabolite CCl_3, a trichloromethyl free radical that binds to lipoprotein and leads to peroxidation of the lipids of the endoplasmic reticulum [16], by the action of the mixed function of the cytochrome P450 oxygenase system. This free radical, which is initially formed as relatively unreactive, reacts very rapidly with oxygen to yield a highly reactive trichloromethylperoxy radical (CCl_3OO). Both radicals are capable of binding to proteins or abstracting a hydrogen atom from an unsaturated lipid, thus, accelerating lipid peroxidation. From the liver enzyme studies we observed that SGOT and SGPT levels were more in case of CCl_4 treated animals because of tissue damage caused by CCl_4 which releases the enzymes in to the blood stream. Upon administration silybin reversed the elevated enzyme levels due to its antioxidant effect.

The reversal of biochemical end points in a CCl_4 hepatotoxic model is better with silybinmicroparticles compared to silybin solution as it offers continuous supply of drug into tissues with no troughs. This suggests that this formulation may be of potential use in the treatment of cirrhosis and fibrosis with silybin. The results can be extrapolated to other drugs suggesting the significant benefits of microparticular passive targeting of drugs to the liver. Similar formulations could not only be used in fibrosis and cirrhosis but also could be used in the liver cancers and several other liver diseases with additional benefits.

CONCLUSION

Silybinmicroparticles can be suitably prepared by emulsion solvent evaporation technique using polycaprolactone as a biodegradable polymer. The particles showed good encapsulationefficiency and sustained drug release. Silybinmicroparticles offer an effective approach for treatment of liver fibrosis.

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