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Original Research Article

Preparation, Characterisation and *In Vivo* Evaluation of Silybin Nanoparticles for the Treatment of Liver Fibrosis

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Abstract

Purpose: To formulate and characterize nanoparticles containing silybin, and evaluate their activity against carbon tetrachloride (CCl₄)-induced liver toxicity.

Methods: Silybin nanoparticles were formulated by o/w emulsion solvent evaporation technique using poly-ε-caprolactone as polymer. Four different nanoparticle formulations (NP₁, NP₂, NP₃ and NP₄) were prepared by varying the drug/polymer ratio. The particles were characterized for particle size, drug content and *in vitro* drug release. The pharmacokinetics and pharmacodynamics of the silybin formulations in male Wistar rats were evaluated following *i.v.* administration, using silybin solution as reference. The hepatoprotective activity of the formulations was also determined in a CCl₄-treated rat model.

Results: Silybin nanoparticles were successfully prepared using o/w emulsion solvent evaporation technique. The nanoparticles sustained the release of the drug both *in vitro* and *in vivo* for up to 10 days and offered better pharmacokinetic properties than the free drug itself. Intravenous nanoparticulate administration reversed serum liver enzyme levels by 95 % compared to only 50 % for the drug solution.

Conclusion: The developed silybin nanoparticles showed superior pharmacokinetic properties and hepatoprotective activity to silybin solution.

Keywords: Silybin, Nanoparticles, Pharmacokinetics, Pharmacodynamics, Hepatoprotective activity.

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INTRODUCTION

Non-alcoholic and alcoholic fatty liver, chronic hepatitis and hepatic carcinoma all of which causes liver fibrosis that results in irreversible cirrhosis ultimately death in human beings [1,2]. Liver fibrosis is the accumulation of extracellular matrix or scar in the liver. There is no standard treatment for liver fibrosis [3]. 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[4].

Silybin is one of the oldest drugs for the treatment of liver fibrosis and cirrhosis [5]. Although it is considered to be ideal for the

treatment of liver fibrosis, delivery to the liver still needs improvement. The effectiveness of oral silybin as a hepatoprotective agent has discounted by its poor solubility, low bioavailability and low half-life [6]. Silybin needs to be administered daily to achieve its effects. Nanosized carriers encapsulating silybin with size less than 6 μm can be taken up passively into Kupffer cells in the liver and can result in increased drug concentration in the liver after intravenous administration, thus increasing therapeutic efficacy. They can result in sustained systemic release of silybin for more than a week, depending on various factors, after forming a depot in the Kupffer cells. Thus, repeated daily administration for silybin can be avoided. Further, oral bioavailability problems with silybin can be avoided since bioavailability is 100 % after intravenous administration.

Oxidative stress in Kupffer cells is known to initiate the formation of liver fibrosis in many diseases and thus antioxidant silybin levels in these cells, if enhanced, can tremendously improve therapy with silybin. Thus, with this type of formulation, sustained parenteral release, improvement in bioavailability as well as enhancement of biochemical protection can be achieved. Together, these mechanisms lead to increase in effectiveness of therapy. Thus, the objective of this study was to prepare biodegradable nanoparticles of silybin, and evaluate their sustained release characteristics, liver targetability and liver protection following intravenous administration.

EXPERIMENTAL

Silybin and Poly- ϵ -caprolactone (mol wt., 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. A probe sonicator (Homogenizer 150 VT), used to prepare the nanoparticles, was procured from M/S Biologics, Inc USA. A zeta sizer (3000 HAS (Malvern Instruments, Malvern, UK) was used to measure the particle size. (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 (Remi Industries, Mumbai, India) was used to facilitate evaporation of dichloromethane while an ultracentrifuge (Remi, Mumbai) was employed to recover the nanoparticles after preparation. Male Wistar rats weighing 150 – 180 g were

purchased from Mahaveer Enterprises, Hyderabad., India

Preparation of silybin nanoparticles

Emulsion (o/w) solvent evaporation method was employed in the preparation of silybin nanoparticles using poly- ϵ -caprolactone as the polymer. Four different nanoparticle formulations NP₁, NP₂, NP₃ and NP₄ containing drug:polymer in the ratio of 1:1, 1:2, 1:3 and 1:4, respectively, were prepared. For the preparation, silybin (100 mg) and polycaprolactone (100, 200, 300 or 400 mg) was dissolved in 15 ml of dichloromethane by vortexing. The mixture (organic phase) was added drop-wise to 50 ml of 2 % PVA solution under probe sonication at 40 w for 12 min to obtain a w/o emulsion. This emulsion was placed on a magnetic stirrer to ensure complete evaporation of dichloromethane, leaving nanoparticle suspension. The suspension was centrifuged at 10,000 rpm for 20 min, resulting in the formation of a pellet at the bottom of the tube. This pellet was washed with phosphate buffered saline (PBS), re-suspended and again centrifuged. The pellet was collected and allowed to dry completely. The powdered particles were collected, weighed and used for further evaluation.

Determination of particle size and charge

The nanoparticles were evaluated for their particle size, polydispersity index of size distribution and surface charge potential, by photon correlation spectroscopy (PCS) using Zetasizer 3000 HAS (Malvern Instruments, Malvern, UK). The formulations were diluted 1:1000 with the aqueous phase of the formulation to obtain suitable kilo-counts per second (kcps). Analysis was performed at 25 °C with an angle of detection of 90°. Each determination was made in triplicate.

Evaluation of encapsulation efficiency

Encapsulation efficiency (EE) was calculated by estimating the amount of untrapped drug. This was found by measuring the absorbance of the drug in supernatant, which was obtained after centrifugation of the nanoparticle suspension and then applying Eq 1.

$$EE (\%) = 100(W_1 - W_2) / W_1 \dots\dots\dots (1)$$

The value obtained from Eq 1 was compared with entrapped drug. For this determination, an accurately weighed amount of nanoparticles was taken in a test tube, dissolved in dichloromethane (DCM) and the solvent allowed

to evaporate completely. An aliquot of methanol (10 ml) was added to the test tube which dissolved only the drug. The absorbance of this solution was measured and the amount of drug encapsulated calculated.

***In vitro* release study**

The *in vitro* release study was performed in a diffusion cell set-up across a dialysis membrane. An inverted cylindrical test tube cut to a height of 8 cm was used as a donor compartment. The receiver compartment consisted of 100 ml of phosphate buffer (pH 7.4, 37 °C) in a beaker placed over a water bath. A dialysis membrane which was pre-soaked in warm water for 30 min was placed at the lower end of the cylindrical set-up and the membrane separated the donor compartment from the receiver compartment. Nanoparticles containing 20 mg of drug was suspended into 5 ml of pH 7.4 buffer and placed in the donor compartment. The system was stirred using a magnetic stirrer and bead. Samples (5 ml) were removed from the receiver compartment and replaced with the same volume of fresh medium immediately. The samples were analyzed spectrophotometrically at 287 nm.

Pharmacokinetic studies

Male Wistar rats (weighing 150 – 180 g each) were purchased from Mahaveer Enterprises, Hyderabad, India, and were maintained in an air-conditioned room at 22 ± 2 °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 [7] 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 [8]. These conditions were maintained throughout the duration of the experiment. The study was performed in three groups of six rats each.

Group 1 received silybin solution containing 30mg/kg intravenously; Group 2 received NP₁ silybin nanoparticles equivalent to 30 mg/kg of drug suspended in normal saline and injected intravenously while Group 3 received placebo nanoparticles. Blood samples were collected at different time intervals over a period of 24 h. For the nanoparticle formulations samples were also

collected days 3, 6 and 9 after administration. Drug levels in the plasma samples were evaluated by HPLC. HPLC standard curve for the drug in plasma was also generated. The UV detection wavelength was 288 nm while the mobile phase consisted of methanol: water (50:50, v/v). The following pharmacokinetic parameters were determined using WinNonlin pharmacokinetic data analysis software: elimination rate constant (k_e), volume of distribution (Vd), elimination half-life ($t_{1/2}$), clearance (CL), and area under curve (AUC).

Evaluation of hepatoprotective activity

Carbon tetrachloride (CCl₄)-induced liver damage model was used in the evaluation of hepatoprotective activity. For this purpose another set of male Wistar rats were divided into five groups each containing 6 rats. Group1 received normal saline (1 ml/rat) daily for 9 days and served as normal control. Group 2 received CCl₄ (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 intravenously daily for 9 days. Group 4 received silybin nanoparticle suspension equivalent to 100 mg/kg of drug intravenously on day 1 while Group5 received placebo nanoparticles. All the groups received CCl₄ 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 3000 rpm for 15 min. Hepatoprotective activity was quantified by serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvic transaminase (SGPT) levels in the plasma. Subsequently, their livers were subjected to histopathological examination. 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.^[13] The body weight of the rats were also monitored.

Statistical analysis

The data were 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

Silybin nanoparticles were successfully prepared using poly- ϵ -caprolactone by o/w emulsion solvent evaporation method. The mean particle size of the nanoparticles ranged from 130 - 430 nm (Table 1). Mean particle size increased with increase in polymer concentration. The polydispersity index (PDI) of the nanoparticles was < 0.4 , indicating that the nanoparticles were homogenous in distribution. The zeta potential value indicates long term stability. The encapsulation efficiency of the nanoparticles increased as polymer concentration increased.

In vitro drug release characteristics

In vitro release data, shown in Fig 1, indicates that all the formulations sustained drug release for over a period of 20 days. A biphasic drug release pattern was found, i.e., burst release followed by sustained release. In the first 6 hours of release, the unencapsulated drug component was released. Subsequently, the encapsulated drug component was gradually released. Drug release from NP1 was higher than from NP2, NP3 and NP4. Log percent cumulative drug released, plotted as a function of log time yielded curves, the slope of is the diffusional release exponent (n). The values of diffusional n were 0.359, 0.374, 0.420 and 0.450 for NP₁, NP₂, NP₃ and NP₄ respectively, which indicate that drug release from all the formulations followed a Fickian pattern [9].

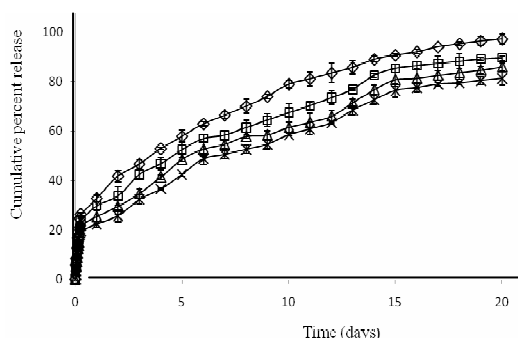


Figure 1: Drug release from silybin nanoparticles (\diamond = NP1, \square =NP2, \triangle = NP3, \times =NP4)

Pharmacokinetics of the formulations

The retention time of the drug, based HPLC studies, was 16.0 and 18.0 min for the two isomers of silybin. The plasma profiles of the drug after administration of nanoparticles and *i.v.* solutions are shown in Figure 2. Nanoparticles resulted in higher and more prolonged drug levels than the drug solution. This was reflected by increased area under the curve. The $t_{1/2}$, AUC and V_d of nanoparticle formulation were higher than for the drug solution, while clearance (CL) was lower than for the free drug (Table 2). All the pharmacokinetic parameters obtained for the nanoparticles were statistically different from those obtained for the drug solution ($p < 0.001$), except C_{max} .

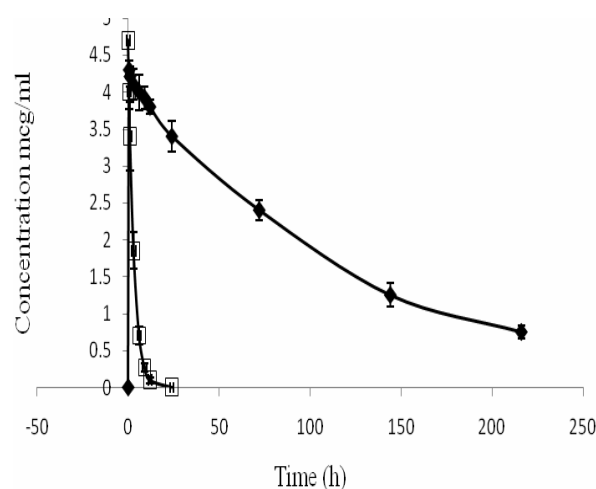


Figure 2: Plasma concentration-time curve of silybin following *i.v.* administration to rats (\diamond = nanoparticles, \square = solution)

Hepatoprotective activity

Table 3 shows hepatoprotective activity data. The administration of CCl_4 to the animals resulted in a marked increase in SGPT and SGOT activities, indicating increased toxicity, but this was mitigated in the animals treated with silybin formulations. The reduction in toxicity was statistically significant at $p < 0.001$ for both silybin formulations and silybin solution.

Table 1: Particle size, charge, PDI and encapsulation efficiency of silybin nanoparticles

Formulation	Particle size (mean \pm SD, nm)	Polydispersity Index(PDI)	Zeta potential (mean \pm SD)	Encapsulation efficiency (%)
NP ₁	135 \pm 3	0.13	-31 \pm 3	91.0
NP ₂	220 \pm 2	0.14	-36 \pm 3	92.6
NP ₃	310 \pm 2	0.20	-34 \pm 3	94.9
NP ₄	426 \pm 1	0.35	-37 \pm 3	95.3

Table 2: Pharmacokinetic parameters for silybin formulations administered i.v. (mean \pm SD, n= 3)

Parameter	Silybin solution by i.v. bolus	Silybin nanoparticles (NP1)
C _{max} (μ g/ml)	4.7 \pm 0.2	4.3 \pm 0.1
Ke (h ⁻¹)	0.316 \pm 0.009	0.00791 \pm 0.00016***
t _{1/2} (h)	2.19 \pm 0.10	87.6 \pm 1.3***
Vd(L)	0.893 \pm 0.04	0.976 \pm 0.007***
Clearance(L/h)	0.282 \pm 0.001	0.00715 \pm 0.00012***
AUC _{0-∞} (μ g.h/ml)	15.42 \pm 0.24	600.8 \pm 0.8***

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, compared with silybin i.v. solution.

Table 3: Effect of silybin formulations on enzyme levels in rats with carbon tetrachloride (CCl₄) -induced hepatotoxicity (mean \pm SD, n = 6)

Treatment Group	Initial body weight (g)	Body weight after 9 days (g)	SGPT(U/L)	SGOT(U/L)
Control	160 \pm 10	180 \pm 15	11.1 \pm 2.3	33.3 \pm 2.9
CCl ₄	165 \pm 15	148 \pm 10	77.8 \pm 3.7	90.0 \pm 3.9
Silybin solution	170 \pm 5	180 \pm 5***	44.4 \pm 4.6***	63.3 \pm 2.8***
Drug nanoparticles	170 \pm 6	196 \pm 15**	18.4 \pm 3.2***	47.6 \pm 4.8***
Placebo nanoparticles	165 \pm 6	156 \pm 12	70.4 \pm 2.3***	86.6 \pm 2.5

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with CCl₄ group

However, the drug nanoparticles completely reversed the elevated levels of SGOT and SGPT.

The photomicrographs in Fig 3 display the histological changes in the liver of the animals following administration of the drug-loaded nanoparticles. The histological profile of the control animals showed normal hepatic architecture with distinct hepatic cells, well-presented cytoplasm sinusoidal spaces and central vein. However, there was disorganization of normal cells with intense centrilobular necrosis following CCl₄ intoxication. Moderate accumulation of fatty lobules and cellular necrosis were observed in the animals treated with silybin solution. However, the nanoparticle formulation exhibited strong protection against CCl₄-induced liver damage, as evidenced by the presence of normal hepatic cords, well-defined cytoplasm and absence of necrosis. Furthermore, the body weights of the rats which fell significantly after CCl₄ treatment were restored to normal following administration of the silybin preparations.

DISCUSSION

In recent years, various new drug carrier systems in the micro- and nanometer size range have been generated to improve drug delivery. Among them, nanoparticles have certain advantages such as a maximal load of the drug and a long shelf life. Furthermore, their body distribution and permeability in tissues can be controlled by size and surface properties [10]. Previously, we demonstrated the enhanced liver protection of curcumin, curcumin analogues and piperine, using kupffer cell (KC)-targeted nanoparticle and liposomal formulations, and sustained release intraperitoneal microspheres [11-13]. Silybin, a natural antioxidant, has long been used for the treatment of chronic liver diseases. The effectiveness of silybin as a liver disease remedy was discounted by its poor solubility and low bioavailability. It offers low bioavailability due to high first pass metabolism, low t_{1/2} and results in patient non compliance due to repeated dosing. Consequently, insufficient concentrations of drugs may accumulate in the target cells. Further, uptake into non-target cells may lead to significant side-effects. Thus we made an attempt to deliver silybin nanoparticles to its target site through bypassing oral route. It can be administered systemically to offer liver protection in a variety of diseases. It can also be targeted to KC and its delivery into these cells can be enhanced with silybin encapsulated in the particulate systems and further improving the therapy.

To achieve the aims of this study, silybin nanoparticles have been prepared using emulsion solvent evaporation technique. Four different formulations NP1, NP2, NP3 and NP4 were prepared. Particle size and entrapment efficiency of silybin nanoparticles increased with increase in polymer content. This may be due to the availability of more polymer to coat the drug. Increase in polymer content also delayed drug release due to increase in particle size and hence reduced surface area available for drug release. Drug release was highest from NP1 due to because it had the polymer content and this has the effect of retarding drug release as a result of increased particle size and reduced surface area available for drug release. Formulation NP1 was used as the optimized formulation for hepatoprotective test because of its lower particle size and polydispersity index (PDI), and good release profile. This particle size favors uptake into all the liver cells responsible for the formation of liver fibrosis [14]. Higher uptake of particles into KC occurs by passive targeting.

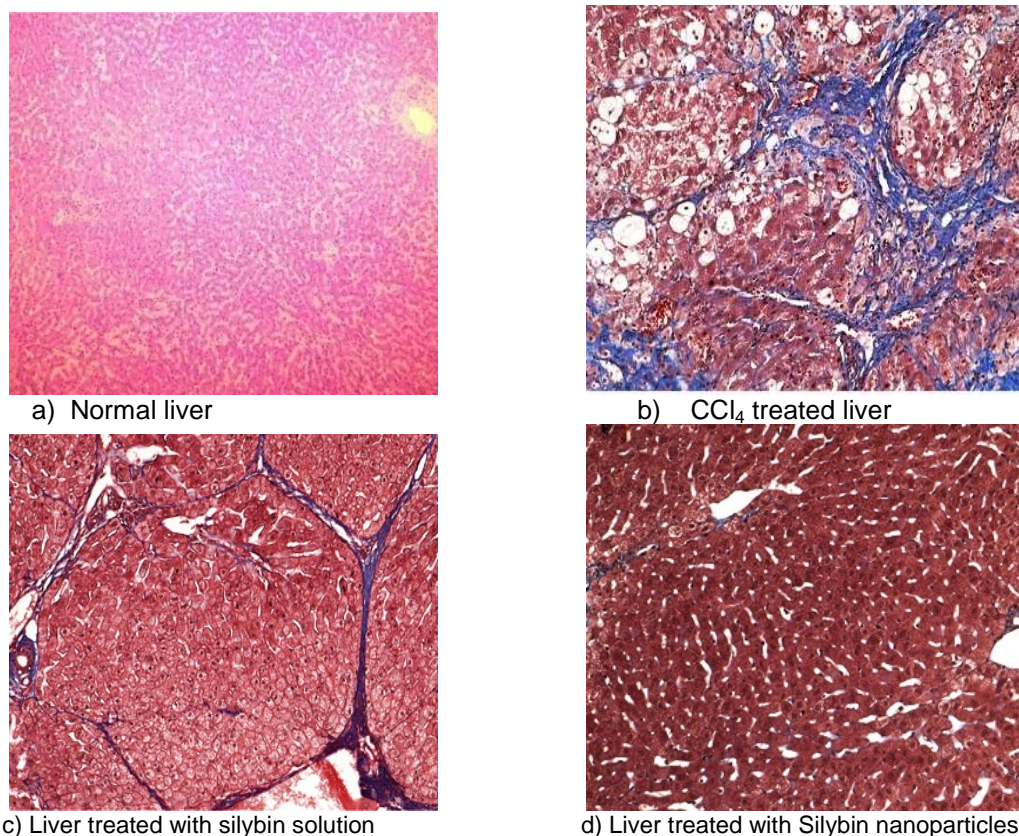


Figure 3: Histopathological profile of rat liver following nanoparticle administration

NP1 sustained drug release for up to 10 days *in vivo* and offered better pharmacokinetic properties than the drug in solution form. Elimination rate constant and clearance than for the drug solution. Furthermore, area under the curve (AUC) was several-fold than for the solution. This suggests reduction in drug elimination and metabolism with nanoparticle formulation administration.

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 CCl₄ is due to its metabolite, trichloromethyl free radical (CCl₃) 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 relatively unreactive when formed initially, reacts very rapidly with oxygen to yield a highly reactive trichloromethyl peroxy radical (CCl₃OO). Both radicals are capable of binding to proteins or abstracting a hydrogen atom from an unsaturated lipid, thus accelerating lipid peroxidation. Expectedly, SGOT and SGPT levels were higher in CCl₄-

treated animals due to tissue damage caused by CCl₄ which results in the release of the enzymes into the blood stream. Upon administration, silybin reduced elevated enzyme levels. Reversal of liver toxicity was greater for nanoparticle formulation than for the drug solution form probably because the nanosized particles were instantly taken up by KC as well as by several other liver cells. As a result, the drug becomes accumulated in the liver cells and is subsequently released at the cellular level to achieve better hepatoprotection.

Overall, the findings of this study suggest that the developed nanoparticle formulation may be useful in the treatment of cirrhosis and fibrosis with silybin. It is likely that these results can be extrapolated to other drugs, suggesting the probability of nanoparticulate passive targeting of drugs to the liver, including for the treatment diseases such as liver cancers.

CONCLUSION

Silybin nanoparticles can be suitably prepared by emulsion solvent evaporation technique using polycaprolactone as a biodegradable polymer. The particles showed good encapsulation efficiency and sustained drug release both *in*

vitro and *in vivo*. Silybin nanoparticles offer an effective approach for drug targeting of the liver.

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