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Silymarin-loaded Eudragit® RS100 nanoparticles improved the ability of silymarin to resolve hepatic fibrosis in bile duct ligated rats

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ABSTRACT

Some nano-formulations of silymarin (SM), a drug commonly used for liver diseases, were developed to overcome its poor solubility and poor bioavailability; antifibrotic effect of these formulations has not been tested yet. In this study we aimed to formulate and evaluate silymarin-loaded Eudragit® RS100 nanoparticles (SMnps) and to test the capability of SMnps to reverse an established fibrosis model. SMnps were prepared by solvent evaporation and nano-precipitation techniques. The influence of drug: polymer ratio, concentration of surfactant in the aqueous phase on particle size, drug entrapment efficiency (EE) % and *in vitro* drug releases were investigated. For *in vivo* evaluation, bile duct ligated (BDL)-rats were treated with either SM or SMnps every other day (125 mg/kg) orally for 3 weeks started 3 weeks after BDL. Liver function tests, oxidative stress and fibrosis/fibrogenesis process were evaluated using biochemical and histopathological techniques. The formulation No (F4) of SMnps showed an average particle size of 632.28 ± 12.15 nm, a drug EE% of 89.47 ± 1.65% and released the drug in a prolonged manner over 24 h. The prepared SMnps were nearly spherical with smooth surfaces. In BDL-rats, treatments with either SM or SMnps corrected liver function and oxidative stress. Only SMnps was able to reverse the induced fibrosis; SMnps significantly decreased serum tumor necrosis factor- α (TNF-α), serum transforming growth factor- β1 (TGF-β1), hepatic hydroxyproline and downregulated the hepatic expression of tissue inhibitor metalloproteinase-1 (*TIMP-1)* and cytokeratin-19 (*CK-19)*, whilst increased hepatic hepatocytes growth factor (HGF) and upregulated the hepatic expression of matrix metalloproteinase-2 (*MMP-2)* and increased *MMP-2/TIMP-1* ratio at mRNA level. Livers of rats treated with SMnps showed very little collagen in ECM and restored hepatic architecture as compared to either BDL rats or rats treated with SM.

Conclusion: Formulation of silymarin as nanoparticles improved its ability to resolve cholestasis-induced liver fibrosis by restoring hepatic regenerative capabilities. Therefore, formulation of SMnps may represent a step forward in the field of anti-fibrotic drug development.

1. Introduction

Several approaches have been reported to improve the *in vivo* performance of poorly soluble drugs when given orally depending on the reduction of drug particle size to increase the dissolution rate and the oral bioavailability of these drugs [1]. Among these approaches, nanotechnology has emerged as a powerful and promising tool. The use of nanoparticulated systems offers many advantages in drug delivery, mainly focusing on improved safety and efficacy of the drugs, providing targeted delivery of drugs, prolonging drug or gene effect in target tissue and improving the stability of drug against chemical/enzymatic degradation [2]. Eudragit® RS100, the co-polymer of poly (ethylacrylate, methyl-methacrylate and chlorotrimethyl-ammoniumethyl methacrylate) containing quaternary ammonium group, is commonly used for the formulation of controlled and sustained release dosage forms [3]. It is insoluble in physiological pH and capable of swelling, which represents a good material for drug dispersion.

Silymarin, an extract of the milk thistle (*Silybum marianum*), is a mixture of flavonoids and polyphenols; Silibinin is its major bioactive constituent. Silymarin is a well-known hepatoprotective drug which has been shown to have antioxidant, anti-

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inflammatory/immunomodulatory, and antifibrotic properties in various *in vitro* and *in vivo* animal models [4]. Its antioxidant activity is most likely to attenuate the pathologic effects initiated by oxidative stress in the liver, which influence pathways of inflammation, necrosis, and fibrosis in chronic liver disease [5,6]. Silymarin is therefore used to treat numerous liver disorders characterised by degenerative necrosis and functional impairment [4]. Silymarin, however, has a very poor aqueous solubility, improper tissue distribution, and gastric degradation [7]. Recent studies developed silymarin in nanoscale were evaluated on paracetamol-induced [8] and CCl_4-induced [9,10] hepatotoxicity. Those and others proved an improvement in pharmacokinetics and bioavailability of silymarin and improvement in liver functions with no attention given to the resolution of fibrosis [8–10]. However, silymarin coated gold nanoparticles promoted extracellular matrix (ECM) degradation, hepatic stellate cells (HSCs) inactivation with strong enhancement of hepatic regenerative capacity in CCl_4-induced liver injury and cirrhosis [11].

Globally, liver fibrosis and cirrhosis constitute a major healthcare Burden. It results from excessive accumulation of ECM proteins including collagen caused by ongoing inflammation and liver cell death that occurs in most types of chronic liver diseases injury including metabolic, viral, cholestatic and genetic disease. Although recent improvements in the treatment of liver diseases, fibrosis remains a long-lasting problem. Experimental and clinical investigations point out that fibrosis is no longer considered irreversible, but the result of a continuous remodeling process [12–14]. Either collagen deposition or resolution takes place depending on the balance between ECM proteinases (matrix metalloproteinases; MMPs) and tissue inhibitor metalloproteinases (TIMPs) [15,16].

With no currently approved drugs for the treatment of liver fibrosis in humans, research is focusing on developing effective anti-fibrotic drugs or even improving the pharmacokinetics of some available drugs like silymarin. The aim of the present work was to (i) formulate and evaluate silymarin-loaded Eudragit® RS100 nanoparticles by solvent evaporation and nano-precipitation method (ii) investigate whether treatment with the newly formulated silymarin nanoparticle can improve the capability of SM reversing cholestasis-induced fibrosis in rats and the underlying mechanism.

2. Materials and methods

2.1. Formulation of silymarin nanoparticle

2.1.1. Materials

Silymarin (SM), Eudragit® RS100 (ERS100) and polyvinyl alcohol (PVA) were gift samples kindly supplied by Sigma Pharmaceutical Industries (Nasr City, Cairo, Egypt), Rohm Pharma (Darmstadt, Germany) and the Egyptian International Pharmaceutical Industries Co.; EPICO (El-Asher of Ramadan city, Egypt), respectively. Dichloromethane and ethanol were purchased from El-Gomhorea Chemical Company (Cairo, Egypt). All other chemicals were obtained from El-Nasr Pharmaceutical Chemical Co., Cairo, Egypt.

2.1.2. Preparation of silymarin nanoparticles

SM-loaded polymeric nanoparticles (SMnps) were prepared by solvent evaporation and nano-precipitation techniques with certain modifications [8,17]. Different formulations of SMnps prepared by using ERS100 as a polymer with different drug: polymer ratio (1:1, 1:2, 1:3, 1:4, 1:5, 2:1 and 3:1). The required amount of drug and polymer were dissolved in a mixture of ethanol and dichloromethane in (1:1) ratio, 5 ml of each with magnetic stirrer *(Heating Magnetic Stirrer–AREC, VELP Scientifica, Innovative Analytical Instruments, EUREOP)*. This solution was added drop wise to 100 ml of an aqueous phase of PVA solution; the contents were allowed to mix for 5 min with homogenizer *(T25 digital Ultra Turax by IKA, Germany)* at 18,000 rpm. 50 ml of distilled water was added as a non-solvent for nano-precipitation. The prepared solution was kept for solvent evaporation under rapid stirring at 10,000 rpm with a mechanical stirrer *(Heidolph PZP-200, Germany)* for 24 h until the organic solvent mixture was evaporated [18]. Nanoparticles were separated by using cooling centrifuge (14,000 rpm for 30 min), supernatant were removed and nanoparticles washed 3 times with distilled water and dried at room temperature in a vacuum desiccator and preserved at 4 °C until further experiments. The prepared nanoparticles were evaluated for percentage yield, drug content, entrapment efficiency%, drug release and characterized for particle size and surface morphology. Different formulations were prepared as shown in Table 1.

2.1.3. Percentage yield

The percentage yield of the prepared nanoparticles was calculated according to the following formula [19].

$$\% \text{ Yield} = (\text{Amount of nanoparticles/Total amount of drug and polymer}) \times 100$$

2.1.4. Scanning electron microscopy (SEM)

The scanning electron microscopy *(SEM) (JEOL JSM-5400LV Jeol, Tokyo, Japan)* was used to analyze the nanoparticles shape and surface morphology (roundness, smoothness, and formation of aggregates). Completely dried particles were taken on aluminum stubs using adhesive tapes and coated with metal using sputter coater then observed for surface morphology under low vacuum at an acceleration voltage of 20 kV [20].

2.1.5. Particle size analysis

Particle size was determined by laser diffraction particle size analyzer (*Mastersizer 2000 Ver. 5.22, Malvern instruments Ltd., UK*) [21,22]. Samples were measured in distilled water as dispersant medium containing 2% w/v of tween 80, to prevent nanoparticles aggregation. The above suspension was sonicated in water bath and the particle size was expressed as volume mean diameter in micrometer using laser diffraction technique.

2.1.6. Drug content determination

To determine the drug content, about 50 mg of the prepared SMnps were added to 10 ml of ethanol to facilitate the coat of the nanoparticles (ERS 100) to get dissolved. This solution was subjected to solvent evaporation for further removal of the solvent prior to filtration by stirring for 15 min at 700 rpm by using magnetic stirrer (Type MM5, Poland). Then the residue was agitated with PBS (pH 7.4) in mechanical shaker (Julabo SW-20C, Germany) for 6 h then kept for 24 h at 25 ± 1 °C. The resultant

Table 1
The composition of different silymarin-loaded nanoparticles formulations.

Formulation No	Drug: polymer ratio	Surfactant conc. (% w/v)
1	1:1	0.5
2	1:2	0.5
3	1:3	0.5
4	1:4	0.5
5	1:5	0.5
6	2:1	0.5
7	3:1	0.5
8	1:4	1.0
9	1:4	1.5