RESEARCH ARTICLE

# Preparation, Characterization and Cytotoxicity of Silibinin-Containing Nanoniosomes in T47D Human Breast Carcinoma Cells

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## Abstract

**Background: Breast cancer is one of the most frequent cancer types within female populations. Silibinin is a chemotherapeutic agent ative against cancer. Niosomes are biodegradable, biocompatible, safe and effective carriers for drug delivery. Objective:To prepare nanoniosomal silibinin and evaluate its cytotoxicity inthe T-47D breast cancer cell line. Materials and Methods: Niosomes were prepared by reverse phase evaporation of a mixture of span 20, silibinin, PEG-2000 and cholesterol in chloroform and methanol solvent (1:2 v/v). The solvent phase was evaporated using a rotary evaporator and the remaining gel phase was hydrated in phosphate buffer saline. Mean size, size distribution and zeta potential of niosomes were measured with a Zetasizer instrument and then nanoparticles underwent scanning electron microscopy. The drug releasing pattern was evaluated by dialysis and the cytotoxicity of nanoniosomes in T-47D cells was assessed by MTT assay. Results: Particle size, size variation and zeta potential of the niosomal nanoparticles were measured as 178.4 ± 5.4 nm, 0.38 ± 0.09 and -15.3 ± 1.3 mV, respectively. The amount of encapsulated drug and the level of drug loading were determined 98.6 ± 2.7% and 22.3 ±1.8%, respectively; released drug was estimated about 18.6±2.5% after 37 hours. The cytotoxic effects of nanoniosome were significantly increased when compared with the free drug. Conclusions: This study finding suggests that silibinin nanoniosomes could serve as a new drug formulation for breast cancer therapy.**

**Keywords:** Silibinin - nanoniosomes - breast cancer - T-47D cells

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## Introduction

Breast cancer is the fifth factor of cause of death among the worldwide women; it is a complex multifactorial disease by genetic and environmental factors and among the factors that increase cancer risk could name age, gender, family history, obesity, diet, race and etc. Although many risk factors for breast cancer are known, its etiology is not known yet precisely (Armstrong et al., 2000; Singletary, 2003). The most common methods for treating breast cancer include surgery, radiotherapy, chemotherapy, hormone therapy and etc. (Coley, 2008). Although the most effective chemotherapy treatments for cancer are toxicity effects of chemotherapy, they may have side effects include liver and kidney damage, immunosuppressant, vomiting, hair loss and etc. A damage normal tissue by tumor drugs is the most important limitations for people with breast cancer, however (Wang et al., 2011; Larsen, 2008).

Targeted drugs deliver to target sites for drug delivery systems to reduce toxicity and improve the therapeutic dose lack of therapeutic effect (Williams et al., 2003). Niosomes are non-ionic surfactant vesicles that formed upon combining non-ionic surfactant of the alky1 or dialkyl polyglycerol ether class with cholesterol. Niosomal drug delivery is potentially applicable to many pharmacological agents for their action against various diseases; it can also be used as vehicle for poorly absorbable drugs to design the novel drug delivery system. Silibinin, is the major polyphenolic flavonoid extracted from the milk thistle Silybum Marianum, and has a wide range of pharmacologic effects. Studies have suggested that silibinin has anti proliferative and anti-cancer effects in various cancer cell lines (Bhatian et al; 1999; Sharma et al., 2002; Lin et al., 2012; Verschoyle et al., 2008; Cho et al., 2013) and this shows that silibinin is a potential drug to treat illnesses related to different types of cancer. However, its efficacy has been extremely restricted due to its poor

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aqueous solubility (Sun et al., 2008). Thus, a large amount of silibinin is required to achieve the therapeutic dose and for overcoming this problem, a variety of delivery systems have been developed in order to improve its solubility and by that its bioavailability, for example, formulations that are using stealth solid lipid nanoparticles (Zhang et al., 2007), mixed micelles (Yu et al., 2010), micro emulsion (Wei et al., 2012), nano suspensions (Wang et al., 2010), porous silica nanoparticles (Cao et al., 2013), liposomes (El-Samaligy et al., 2006), and Nano/micro hydrogel matrices (El-Sherbiny et al., 2013), as drug carriers have been extensively employed for the enhancement of bioavailability of the poorly water soluble drug silibinin.

Different Nano-carriers have been used for delivering silibinin, but researchers have to be successful yet in producing the appropriate Nano niosoaml silibinin formulation. In this research, Nano niosoaml silibinin were optimized and their toxic effects were assessed on breast cancer cell line.

## Materials and Methods

Silibinin, span 20, Cholesterol, polyethylene glycol 2000 (PEG-2000), and MTT (0.5 mg/ml) were obtained from Sigma company; the RPMI-1640 culture medium was purchased from Invitrogen (Invitrogen, USA) and T-47D cell line was supplied by Pasteur Institute of Iran.

*Preparation of Niosomes*

Niosomes were prepared by reverse phase evaporation method. Span 20, PEG-2000, cholesterol and silibinin (400:55:50:100 mg) were dissolved in 20 ml of chloroform and methanol solvent (1:2 v/v) (300 rpm, 3h, room temperature) to gain a transparent, uniform suspension. Then solvent phase of the resultant solution was removed by using rotary evaporator (Heidolph, Germany, 90 rpm, 1 h, and 50ºC) and then, dried under nitrogen gas. After that, 20 ml phosphate buffer (pH 7.4, 20mM) was added to the resulting gel lose phase and was stirred (300 rpm, 3 h, room temperature); a control formulation was also prepared without drug. Finally, the formulation were sonicated (Bandelin Sonorex Digitec, 60 HZ) for 5 minutes to reduce the size of liposomes and enhancement of homogeneity (Rostas and Dyess, 2011).

*Characterization of niosomes*

Mean size, size distribution and zeta potential of particles were determined by Zetasizer (Nano ZS3600, Malvern Instruments of UK). Morphology and homogeneity of NPs were studied by scanning electron microscope (XL30, Philips, Netherlands).

In vitro study of drug release

*Drug release was determined by dialysis method; one* ml of each formulation (test and control) were poured into dialysis bags (cut off:8 kD, Sigma) and put into 25 ml phosphate buffer (pH 7.4, 20mM) and stirred (100 rpm, 37 h, room temperature). At predetermined time intervals, 2 ml of phosphate buffer was taken and then substituted by fresh phosphate buffer. Finally, the amounts of released silibinin in phosphate buffer were measured by spectrophotometer at 290 nm and the amount of released drug was estimated by the standard curve.

*Determination of encapsulation efficiency and drug loading*

For this purpose, 2 ml of niosomal drug (contains a 10 mg of silibinin) and its control, were centrifuged at 16000 rpm for 45 min at 15ºC. The clear supernatant was removed, and the precipitate was washed three times with PBS to remove un-entrapped drug; the precipitate was then dissolved in equal volume of triton X-100 (0.5%) and vortexed for 10 min. After centrifugation, the optical density of the upper phase was measured by spectrophotometer (UV-160IPC, Shimadzu, Japan). The amount of un-entrapped drug was calculated by the following formulas and using standard curve.

$$\text{Encapsulation (\%)} = \frac{(\text{Amount of drug in carrier(mg/ml)})}{(\text{Amount of drug fed initially (mg/ml)})} \times 100 \quad \text{(Formula 1)}$$

$$\text{Loading efficiency (\%)} = \frac{(\text{Amount of drug in nanoparticle (mg/ml)})}{(\text{Weight of nanoparticle (mg/ml)})} \times 100 \quad \text{(Formula 2)}$$

To obtain respected standard curve, several dilutions of silibinin were prepared and their optical density were measured at 290 nm wavelength and the optical density against concentration was depicted by using Excel program.

*Cytotoxicity assay*

Viability test was determined by MTT assay on T-47D cell line and cells were cultured in a 96-well plate at a

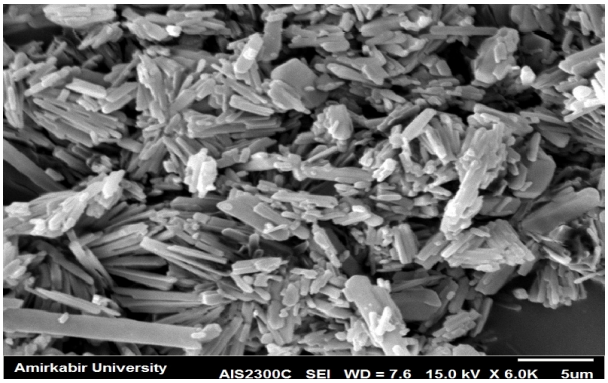


**Figure 1. SEM Image of the Silibinin Nanoniosomes**

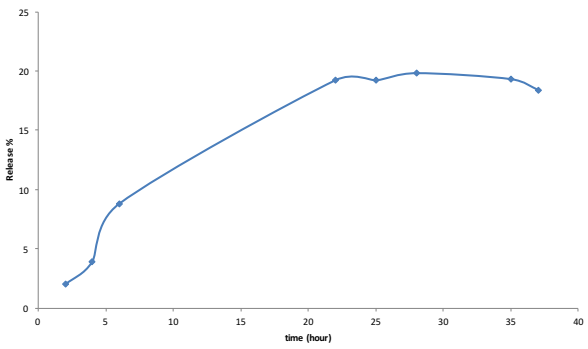


**Figure 2. In vitro Release Profile of Silibinin from Niosomal Formulation in Phosphate Buffered Saline (pH 7.4).**

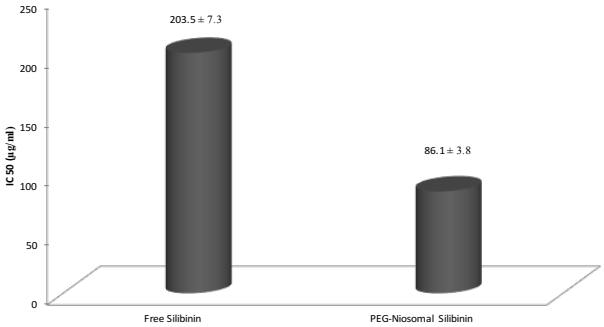


**Figure 3. The IC50 of Niosomal and Free Silibinin against T-47D Cell Line during 48h Incubation**

density of $1\times10^4$ (10,000 cells) and cultivated with 5% $CO_2$ at 37°C in RPMI-1640 culture medium containing 10% fetal bovine serum and 1% penicillin/ streptomycin antibiotics; they were allowed to attach for 24h. After removing supernatant, cells were treated with free silibinin and niosomal silibinin in different concentrations. Viability was evaluated during 48 h incubation and absorbance was measured at 570 nm by Elisa reader (BioTek Instruments, VT, of USA). IC50 amount was determined by statistical package Pharm-PCS software (Springer Verlag, USA).

*Statistical analysis*

The results are expressed as mean ± standard deviation (SD, n = 3). The data were statistically analyzed by one-way analysis of variance using IBM Statistics SPSS software version 19, and statistical significance was set at $p < 0.05$.

## Results

*Characterization of niosomes*

The size, poly dispersity index (PDI) and zeta potential of niosomes were obtained, 178.4 ±5.4 nm, 0.38±0.09 and -15.3±1.3 mV, respectively. Scanning electron microscopy (SEM) indicated that the nanoparticles had a cylindrical shape and monodispers pattern (Figure 1).

*In vitro study of drug release*

The releasing of silibinin from niosomal vesicles in phosphate buffer (pH 7.4, 20mM), was determined within 2, 4, 6, 8, 19, 31, and 37 hours (Figure 2). This study finding indicated that about 18.6 ± 2.5 % of the drug was released during 37 h incubation.

*Determining drug loading and encapsulation*

The encapsulation percentage was calculated according to the standard curve for drug formulation. Using the two formulas, the percent of encapsulation and loading efficiency were 98.6±2.7% and 22.3±1.8%, respectively.

*In vitro viability*

The in vitro viability test of niosomal silibinin and free drug were surveyed by MTT assay in T-47D cells. The half maximal inhibitory concentration (IC50) of niosomal silibinin and free drug for T-47D is illustrated in Figure 3. It was shown that both free drug and PEG-niosomal silibinin exhibited clear dose-dependent cytotoxicity against this cell line, but the efficacy of niosoaml drug against tumor cells was better than free drug.

## Discussion

In this research, niosomal silibinin was conducted to optimize and assess the toxic effects of silibinin Nano niosomes on breast cancer cell line; results of the research showed an increase in cytotoxic effects of silibinin loaded on the niosomes compared with free form of silibinin. However, nanoniosome synthesis techniques have proved beneficial in improving of drug in the target site (Zarei et al., 2013). Nano drug delivery has been proven in a study at 2001 by Fang and Et al. that loaded Anozation drug to noisome and in the presence of cholesterol increases the drug's positive characteristics (Fang et al., 2001). In this study, the pegylated niosomal silibinin was prepared by high entrapment and loading efficiency; the experiments performed as triplicate. Results showed that trial had sufficient validity and reproducibility and the results of the particles diameter measurements by using zetasizer device after their fabrication confirmed the particle size in the Nano scale (Zhang and Feng, 2006). The roles of various drugs with Nano niosomal formulations were evaluated in different cell lines, for instance, Mohammad Zarei at al. (Zarei et al., 2013), investigated the toxic effects of niosomal paclitaxel in breast cancer cell line. Drug release showed the amount of paclitaxel released from nano-particles (niosomes) within 48 hours at about 6 %. Their investigations also showed that the toxic effect of Nano niosoaml paclitaxel increased when compared with paclitaxel (free) drug and it was found that the niosomes had a high drug retention capability so that about 18% of drug was released after 37 hours. Release study showed that release process had a steady state without burst effect and it was probable that presence of polyethylene glycol had led to low level of release (Mansour et al., 2009). Moreover, size was also an important factor that determined the release rate and the nanoparticles prepared by this method had an appropriate size (Astete and Sabliov 2006).

The cytotoxic effect of niosomal silibinin formulations was carried out by MTT assay in which the formulation without drug (control) did not represent any cytotoxicity effect on T-47D cells. The results indicated that the least $IC_{50}$ belonged to niosomal silibinin was less than that of free silibinin; this phenomenon seemed to be due to the effect of PEG on more stability and slower drug releasing of niosomal formulation. Also, PEG increased drug solubility and its collision with the targeted cell.

In conclusion, Taking collectively, this research confirms that Nano niosomal silibinin has more cytotoxic effects than free silibinin on breast cell line; thus, this formulation may be an alternative chemotherapeutic candidate for breast cancer in the future.

## Acknowledgements

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