

## NIOSOMES AS A NOVEL PHARMACEUTICAL FORMULATION ENCAPSULATING THE HEPATOPROTECTIVE DRUG SILYMARIN

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### ABSTRACT

Silymarin is a purified extract isolated from seeds of the milk thistle *Silybum marianum*. It has been used for more than 2000 years to treat liver and gallbladder disorders. Based on the poor bioavailability of silymarin and on the advantages of niosomes, the objective of this research is to develop a silymarin niosomal preparation with enhanced activity and limited side effects. Silymarin loaded niosomes were prepared using different non-ionic surfactants (NIS), cholesterol (Ch) and different charge inducing agents (CIA) in molar ratios (1:1:0.1) and (2:1:0.25). The effect of components molar ratio and effect of surface charges on the percentage drug encapsulated were investigated. Characterization of prepared niosomes was performed via transmission electron microscopy (TEM), differential scanning calorimetry (DSC), particle size analysis and also investigation of the *in-vitro* release profiles. Selected silymarin niosomal formulations were evaluated for their hepatoprotective activity against carbon tetrachloride (CCl<sub>4</sub>) induced oxidative stress in albino rats. Biochemical parameters like serum glutamate oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase (SALP) were used to measure the degree of liver protection. Silymarin niosomal formulations produced a significant decrease in both transaminase levels as well as in SALP level in comparison with administered silymarin suspension. This improvement was also proven histopathologically.

**Keywords:** Niosomes, Silymarin, Hepatoprotective, Biochemical, Histopathology.

### INTRODUCTION

Over the past three decades, significant advances have been made in drug delivery technology. Drug delivery system (DDS) is an important component of drug development and therapeutics<sup>1</sup>. The low cost, greater stability and ease of storage of non-ionic surfactants led to the exploitation of these compounds as alternative to phospholipids, the main constituent of liposomes<sup>2</sup>.

Niosomes are microscopic lamellar structures formed on admixture of a non ionic surfactant, cholesterol and a charge inducing agent with subsequent hydration in aqueous media<sup>3 and 4</sup>. Niosomes possess an infrastructure consisting of hydrophobic and hydrophilic moieties together and as a result can accommodate drug molecules with a wide range of solubilities<sup>5</sup>. Niosomes have been evaluated in many pharmaceutical applications. In such therapeutic applications, important advantages of using niosomes include their ability to reduce systemic toxicity by encapsulation of treatment agents and minimize clearance of such agents from the body by slow drug release<sup>6</sup>.

Silymarin is a polyphenolic flavonoid isolated from seeds of the milk thistle *Silybum marianum* (Family Asteraceae). It has been used to treat liver and gallbladder disorders, including hepatitis, cirrhosis, and jaundice, and to protect the liver against poisoning from chemical and environmental toxins, including snake bites, insect stings, Amanita phalloides mushroom poisoning, and alcohol<sup>7-9</sup>. Silymarin has also been reported to provide liver protection against CCl<sub>4</sub> and paracetamol-induced liver damage in rat models<sup>10-11</sup>.

Silymarin's effects are accomplished via several mechanisms. It prevents lipid peroxidation<sup>12</sup>, protects the cell membrane from radical-induced damage<sup>13</sup>, blocks the uptake of toxins such as Amanita phalloides toxin<sup>14-15</sup> and stimulates ribosomal RNA polymerase thereby increases protein synthesis<sup>16</sup>. Other mechanisms include anti-inflammation<sup>17</sup>, antifibrosis<sup>18</sup> and anticarcinogenesis<sup>19</sup>.

Silymarin absorption rate levels vary between 20 and 50%<sup>20</sup>. Several reasons have been attributed for this poor bioavailability, e.g., poor enteral absorption<sup>21</sup>, degradation by gastric fluid<sup>22</sup> or its poor solubility<sup>23-25</sup>. Several pharmaceutical approaches have been employed to improve the bioavailability of silymarin. These approaches include complexation of silymarin with phosphatidylcholine (Siliphos)<sup>26-28</sup>, complexation with cyclodextrins<sup>29</sup>, complexation with phospholipids<sup>24</sup>, provision of silymarin in the

form of salts of polyhydroxyphenyl chromanones<sup>23</sup> and other more soluble derivatives<sup>30</sup>.

Based on the successes and the advantages of niosomes, and the poor bioavailability of silymarin, the objective of this research is to develop silymarin niosomal preparation with enhanced activity, extended over a prolonged period and with limited side effects.

### MATERIALS AND METHODS

#### Materials

Silymarin was kindly supplied by Medical Union Pharmaceutical Co. Abu Sultan (Egypt). Sorbitan monostearate (Sp 60) was purchased from Merck Schuchardt OHG (Germany). Sorbitan monopalmitate (Sp 40), Cholesterol (Ch) and Dicyetyl Phosphate (DCP), Sigma Aldrich Co. (Germany). Stearyl amine (SA), Sigma Chemical Co. (USA). Methanol, Alliance Bio. California (USA). Chloroform, RPS Chemicals Co., London, (England). Sodium chloride, Potassium dihydrogen phosphate, disodium hydrogen phosphate and Propylene glycol, Oxford-Laboratory, Mumbai (India). Heavy Liquid Paraffin, ElNasr Pharmaceutical Chemicals Co., Abu Zaabal, (Egypt). Carbon tetrachloride (CCl<sub>4</sub>), Alpha Chemicka, Mumbai (India)

#### Preparation of niosomes

Plain niosomes and silymarin niosomes were prepared using the Hand Shaking Method<sup>31</sup>. Accurately weighed quantities of the drug, the non-ionic surfactant (either Sp 40 or Sp 60) and Cholesterol (either alone or mixed with a CIA) in different molar ratios were dissolved in chloroform/methanol mixture (1: 1, v/v) in a round-bottom flask<sup>32</sup>. The organic solvents were slowly evaporated under reduced pressure using the rotary evaporator, at 58-60°C<sup>32-33</sup>. After evaporation of the organic solvents, the thin film formed on the inner wall of the rotating flask is then hydrated with 10 ml phosphate buffered saline (PBS) pH=7.4 pre-warmed to 58°C-60°C. The drug containing niosomes were separated from un-entrapped drug by cooling centrifugation of the niosomal formulation using 5400 xg at -4°C. The pellets formed were washed twice with phosphate buffered saline (pH=7.4) and re-centrifuged again for 30 min.

#### Determination of Silymarin Entrapment Efficiency in Niosomes

The concentration of the entrapped drug was determined by lysis of the niosomal pellet with methanol and sonication to obtain a clear

solution<sup>34-35</sup>. The concentration of drug in methanol, after filtration using 0.45 µm Millipore filter, was determined spectrophotometrically by measuring the U.V. absorbance at  $\lambda = 288\text{nm}$  which is the maximum absorption of silymarin in methanol<sup>36</sup>. Further dilution was made if necessary. The encapsulation or entrapment efficiency was calculated relative to the original drug amount through the following relationship: Entrapment efficiency percentage (E %) =  $ED/TD \times 100$ , where ED is the amount of encapsulated drug and TD is the total amount of drug added<sup>37</sup>.

The following factors affecting entrapment efficiency were investigated:

**a) Effect of niosomal surface charge on the percentage of drug entrapped**

Surface charges were imparted to drug niosomal preparations using charge inducing agents. Charge inducers are used to impart charge on the vesicles to increase its stability by preventing fusion of vesicles<sup>38</sup>. For inducing a negative charge, DCP was added while for inducing a positive charge, SA was incorporated.

**b) Effect of niosomes components molar ratio on the percentage of drug entrapped**

Two molar ratios were used for the preparation of negatively and positively charged niosomes, namely; NIS: Ch: CIA (1:1:0.1) and (2:1:0.25).

**Transmission Electron Microscopy (TEM)**

All niosomal systems prepared, were examined under TEM. A drop of the niosome sample was transferred into the copper mesh grids. After the sample was adsorbed (about 15~20 min), the staining dye (potassium phosphotungstate) was dripped onto the film. The staining time was about 1~2 min<sup>39</sup>. After drying the copper mesh grids, the morphology of the investigated niosomes was clearly observed by transmission electron microscopy.

**Differential Scanning Calorimetry (DSC)**

DSC was carried out for silymarin powder as well as for the dehydrated pellets of the niosomal formulations. The apparatus employed for the thermal analysis was Shimadzu-DSC 50 Differential Scanning Calorimeter. Computer presentations of the DSC thermograms were provided using the same apparatus. The temperature scan ranged from 20°C to 120°C with a scan rate of 5°C/min. The analysis was performed under nitrogen atmosphere using aluminum pans. The weights used for the niosomal preparations were equivalent to 1 mg of the non-ionic surfactant (NIS) investigated, viz, either span60 or span 40.

**Particle Size Distribution Measurements of Niosomal Vesicles**

The particle size of the prepared silymarin vesicles was measured by dynamic light scattering (DLS) based apparatus (NICOMP 380 ZLS, PSS-Nicom Particle Sizing Systems) at the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, (Egypt).

**In-vitro Release Profiles of Silymarin Niosomes**

This experiment was conducted using neutral and negative niosomal formulations. Positively charged niosomes were omitted in this experiment due to the reported toxicity<sup>40</sup> and aggregation<sup>41</sup>. The amount of silymarin entrapped at zero time was considered as the total amount of the drug (100 %). Dilution of the pellets of each preparation was then carried out to exactly 10 ml using PBS (pH 7.4). Thus, the preparations were ready to undergo the hydrodynamic stress conditions (rotation at a rate of 150 strokes/ min and adjusting the temperature to 37°C). One ml sample from each of the niosomal suspensions was taken at different time intervals, namely; at 3, 6, 24, 48 and 72 hours after the start of the experiment. After separation and washing of the samples, the amount of silymarin retained inside niosomal vesicles was determined, at each time interval, spectrophotometrically at  $\lambda_{\text{max}}$  288 nm. The mean amount of silymarin retained was then calculated at each time interval for each of the eight formulations investigated.

**In-Vivo Study on Silymarin Niosomes**

**Experimental animals**

The study was carried out on female Albino Wistar rats weighing 110-150 gm. The animals were housed in clean cages and maintained in controlled temperature ( $23 \pm 2^\circ\text{C}$ ) and light cycle (12 h light and 12 h dark). They were fed with standard diet and water.

**Assessment of hepatoprotective activity**

Animals were divided into six groups each of six rats. Group I was kept as a control group and received only vehicle [(Propylene glycol: PBS (3:1)]<sup>42</sup> via the subcutaneous route (s.c.). Group II acted as toxin control and received vehicle for five consecutive days. Also,  $\text{CCl}_4$  in liquid paraffin (1:1) at a dose of 2ml/kg b.w., intraperitoneally (i.p.) was injected on 4<sup>th</sup> day<sup>43</sup> to induce hepatic damage. Groups III, IV, V and VI received plain niosomal suspension, silymarin suspension (silymarin in vehicle) (100 mg/kg body weight (b.w.)), neutral silymarin loaded niosomes of the molar ratio Sp60: Ch (1:1) (100mg /kg b.w.) (N1) and neutral silymarin loaded niosomes of the molar ratio Sp40: Ch (2:1) (100 mg/kg b.w.) (N2), respectively, via the subcutaneous (s.c) route for five consecutive days, as well as,  $\text{CCl}_4$  in liquid paraffin (1:1), 2ml/kg b.w on 4<sup>th</sup> day intraperitoneally (i.p.). On the sixth day, the blood was collected from the retro orbital plexus of each animal and serum was separated. Collected serum was biochemically tested for transaminase levels of both types i.e. SGOT and SGPT as well as SALP level.

**Histopathological Study**

After collecting the blood from each animal, animals were sacrificed. Liver was immediately separated, fixed in 10% formalin, serially sectioned and microscopically examined after staining with hematoxylin and eosin to analyze any pathological changes.

**Statistical Analysis**

All data are presented as the arithmetic mean values  $\pm$  standard deviation (mean  $\pm$  SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by LSD or independent sample t test using SPSS® software. Difference at  $P < 0.05$  was considered to be significant.

**RESULTS AND DISCUSSION**

**Silymarin Entrapment Efficiency in Niosomes**

Table 1 illustrates the drug entrapment percentages in Sp60 and Sp40 niosomes, prepared using different molar ratios and different surface charges. The prepared silymarin loaded niosomes formed of Sp 60 and Ch, at 1:1 molar ratio, showed promising drug encapsulation efficiency of  $70.61 \pm 1.832$ .

**Effect of surface charge on the percentage of drug entrapped in Sp60 and Sp40 niosomes**

Figures (1 and 2) and table (1) reveal clearly the effect of niosome surface charge on silymarin entrapped percentages for sp60 and sp40 niosomes, respectively. Statistical analysis reveals a significant difference ( $P < 0.001$ ) between neutral and negatively charged silymarin niosomes of the molar ratios Sp 60: Ch (1:1) and Sp: Ch: DCP (1:1:0.1), respectively, where incorporation of the negative charge inducer (DCP) significantly decreased the entrapment percentage from  $70.61\% \pm 1.832$  to  $62.49\% \pm 1.756$ . Also, by the incorporation of the positive charge inducer (SA) has led to a significant decrease ( $P < 0.001$ ) in the percentage of silymarin entrapment to reach  $52.62 \pm 2.07$ . Considering the molar ratio Sp60: Ch (2:1), statistical analysis (Table 1) also, reveals a significant difference between neutral and negatively charged niosomes of the molar ratios Sp 60: Ch (2:1) and Sp 60: Ch: DCP (2:1:0.25), respectively, where incorporation of DCP led to a significant decrease ( $P = 0.025$ ) in entrapment percentage from  $49.14 \pm 1.268$  to  $43.8 \pm 3.202$ . Table I also, show an insignificant difference ( $P = 0.490$ ) between the entrapment percentages of neutral ( $49.14 \pm 1.268$ ) and positively charged silymarin niosomes ( $47.72 \pm 3.418$ ) of the molar ratios Sp 60: Ch (2:1) and Sp 60: Ch: SA (2:1:0.25), respectively.

Considering Sp40 niosomes, statistical analysis of the data (Table 1) reveals a significant difference ( $P=0.006$ ) between neutral ( $61.33 \pm 1.806$ ) and negatively charged niosomes ( $53.78 \pm 3.641$ ) of the two molar ratios Sp40: Ch (1:1) and Sp40: Ch: DCP (1:1:0.1), respectively.

Upon investigating the data of the molar ratio Sp40: Ch (2:1), same conclusions were obtained. Results reveal a significant difference ( $P=0.001$ ) between neutral ( $62.82 \pm 2.321$ ) and negatively ( $56.18 \pm 2.472$ ) charged niosomes of the two molar ratios Sp

**Table 1: Effect of surface charge and cholesterol content on percentage of silymarin entrapped in niosomes prepared using the NIS Sp60 and Sp40**

Molar Ratio (NIS: Ch: CIA)	Mean drug entrapment % $\pm$ S.D.		
	Neutral	Negative	Positive
Sp 60 :Ch: CIA (1:1:0.1)	70.61 $\pm$ 1.832	62.49 $\pm$ 1.756 <sup>a</sup>	52.62 $\pm$ 2.07 <sup>a</sup>
Sp 60 :Ch: CIA (2:1:0.25)	49.14 $\pm$ 1.268 <sup>a</sup>	43.8 $\pm$ 3.202 <sup>b,e</sup>	47.72 $\pm$ 3.418 <sup>f</sup>
Sp 40 :Ch: CIA (1:1:0.1)	61.33 $\pm$ 1.806	53.78 $\pm$ 3.641 <sup>c</sup>	58.93 $\pm$ 3.304
Sp 40 :Ch: CIA (2:1:0.25)	62.82 $\pm$ 2.321	56.18 $\pm$ 2.472 <sup>d</sup>	66.48 $\pm$ 2.697 <sup>g</sup>

Values of '<sup>a</sup>' exhibit significant difference from neutral niosomes Sp 60: Ch (1:1),  $P < 0.05$ .

Values of '<sup>b</sup>' exhibit significant difference from neutral niosomes Sp 60: Ch (2:1),  $P < 0.05$ .

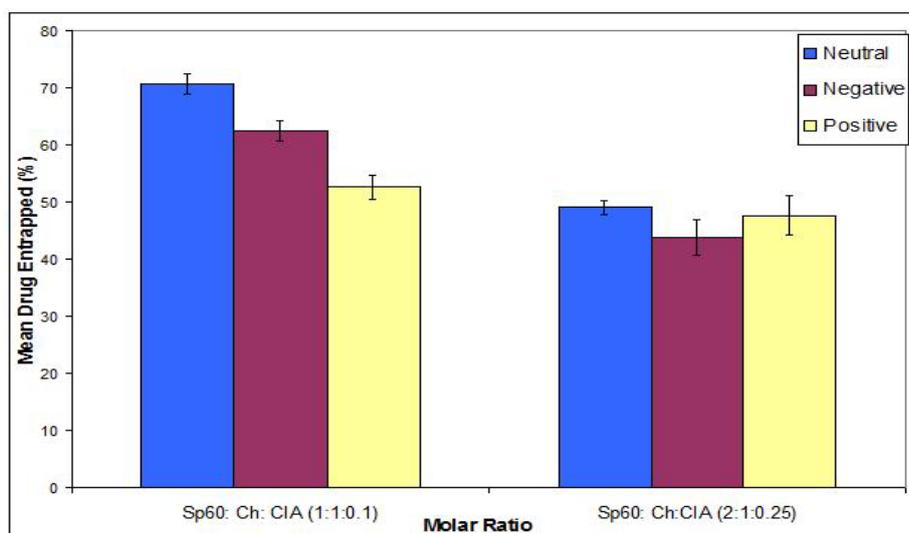
Values of '<sup>c</sup>' exhibit significant difference from neutral niosomes Sp 40: Ch (1:1),  $P < 0.05$ .

Values of '<sup>d</sup>' exhibit significant difference from neutral niosomes Sp 40: Ch (2:1),  $P < 0.05$ .

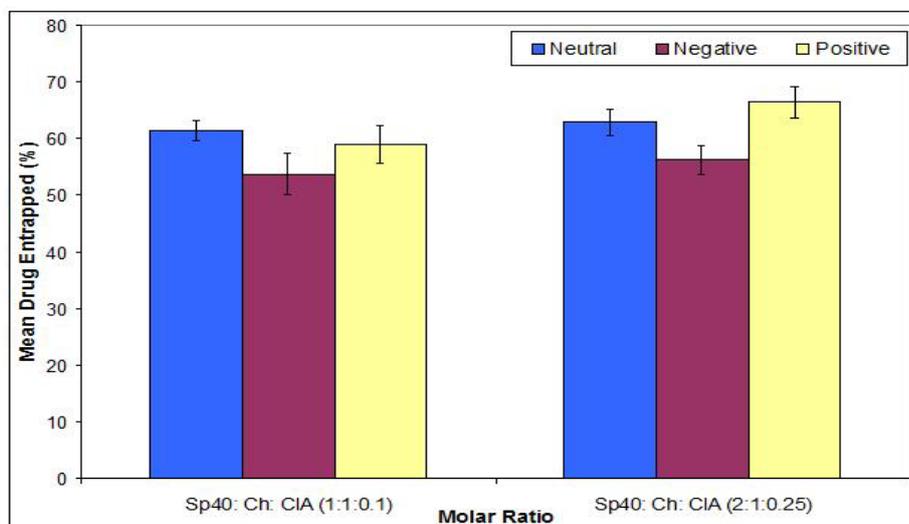
Values of '<sup>e</sup>' exhibit significant difference from negative niosomes Sp 60: Ch: CIA (1:1:0.1),  $P < 0.05$ .

Values of '<sup>f</sup>' exhibit significant difference from positive niosomes Sp 60: Ch: CIA (1:1:0.1),  $P < 0.05$ .

Values of '<sup>g</sup>' exhibit significant difference from positive niosomes Sp 40: Ch: CIA (1:1:0.1),  $P < 0.05$ .



**Fig. 1: Effect of surface charge on percentage of silymarin entrapped in sp 60 niosomes**



**Fig. 2: Effect of surface charge on percentage of silymarin entrapped in sp 40 niosomes**

40: Ch (2:1) and Sp 40: Ch: DCP (2:1:0.25), respectively.

Table 1 reveals the results of incorporation of the positive charge inducer SA, where an insignificant difference ( $P=0.268$ ) was revealed between the entrapment efficiencies of neutral ( $61.33 \pm 1.806$ ) and positively ( $58.93 \pm 3.304$ ) charged niosomes of the molar ratios Sp 40: Ch (1:1) and Sp40: Ch: SA (1:1:0.1), respectively. Same was noticed upon comparing the entrapment percentage of neutral ( $62.82 \pm 2.321$ ) and positively ( $66.48 \pm 2.697$ ) charged niosomes of the molar ratios Sp 40: Ch (2:1) and Sp 40: Ch: DCP (2:1:0.25), respectively, where the difference between them appeared to be insignificant ( $P=0.051$ ).

#### Effect of niosome components molar ratio on the percentage of drug entrapped in Sp60 and Sp40 niosomes

Figures 3 and 4 reveal the effect of niosomes components molar ratios on the amount of drug entrapped in Sp60 and Sp40 niosomes, respectively. Table I reveals the statistical analysis of

the data, indicating that increasing the amount of cholesterol content from 33% in the molar ratio Sp 60: Ch (2:1) to 50% in the molar ratio Sp 60: Ch (1:1) significantly increased ( $P<0.001$ ) the drug entrapment efficiency from  $49.14 \pm 1.268$  to  $70.61 \pm 1.832$ . Regarding negatively and positively charged Sp 60 niosomes, same was noticed. Increasing cholesterol content from 33% in the molar ratio Sp 60: Ch: CIA (2:1:0.25) to 50% in the molar ratio Sp 60: Ch: CIA (1:1:0.1) significantly increased the drug entrapment efficiency from  $43.8 \pm 3.202$  to  $62.49 \pm 1.756$  ( $P<0.001$ ) and from  $47.72 \pm 3.418$  to  $52.62 \pm 2.07$  ( $P=0.049$ ) for negatively and positively charged niosomes, respectively. Cholesterol alters the fluidity of chains in bilayers and, when present in sufficient concentration, abolishes the gel to liquid phase transition of surfactant bilayers<sup>3, 33 and 44</sup>. It also increases the microviscosity of niosomal membrane conferring more rigidity<sup>45</sup>.

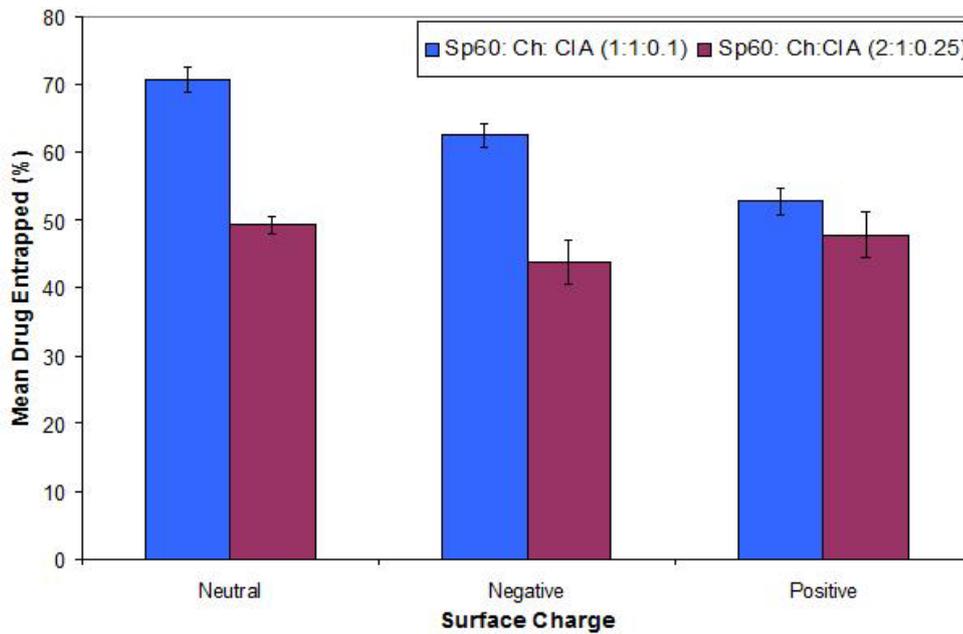


Fig. 3: Effect of niosomes components molar ratio on percentage of silymarin entrapped in sp60 niosomes

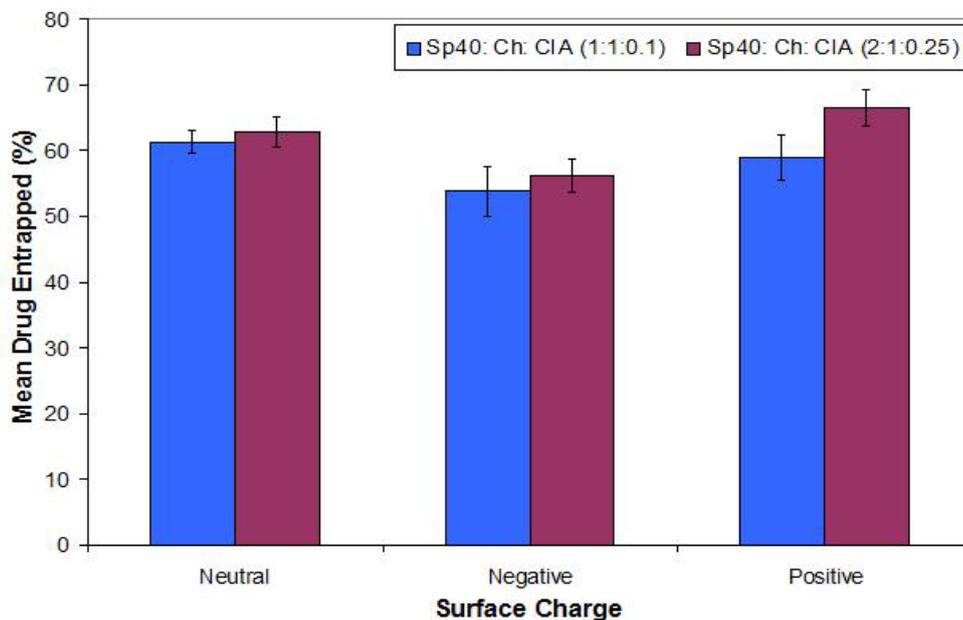


Fig. 4: Effect of niosomes components molar ratio on percentage of silymarin entrapped in sp40 niosomes

Considering neutral and negatively charged silymarin niosomes prepared using the NIS Sp 40, Statistical analysis of the data, reveals an insignificant difference ( $P > 0.05$ ) between the drug entrapment efficiencies of the molar ratios Sp 40: Ch: DCP (2:1:0.25) and Sp40 Ch: DCP (1:1:0.1). Regarding the positively charged silymarin Sp40 niosomes, statistical analysis reveals a significant difference ( $P = 0.008$ ) between the drug entrapment efficiencies in positively charged niosomes prepared using the molar ratios Sp 40: Ch: SA (1:1:0.1) and Sp 40: Ch: SA (2:1:0.25). Thus, we can conclude that the effect of increasing surface charge inducer content in the molar ratio Sp 40: Ch: SA (2:1:0.25) overcame that of the higher cholesterol content in the molar ratio Sp 40: Ch: SA (1:1:0.1). Increasing SA content significantly increased the drug entrapment efficiency from  $58.93 \pm 3.304$  to  $66.48 \pm 2.697$ .

#### Characterization of prepared silymarin niosomes

##### Transmission Electron Microscopy (TEM)

Figures 5 and 6 show selected micrographs prepared using different surface charges and different molar ratios. As observed, the micrographs reveal the spherical shape and the bilayered structure of the prepared niosomes that exist in disperse or in aggregate

collections.

##### Differential Scanning Calorimetry (DSC)

DSC thermograms of silymarin, plain (drug-free) niosomes of the molar ratio Sp 40: Ch (2:1) as well as silymarin loaded niosomes of the same molar ratio, are illustrated in Figure 7.

A DSC thermogram of silymarin showed an endothermic peak at  $218.8^\circ\text{C}$ . Plain (drug-free) and drug loaded niosomal formulations showed broad transitions which are characteristic for lipid mixtures containing cholesterol, signifying good interaction of all components forming the bilayers of niosomes<sup>46</sup>. A DSC thermogram of plain neutral niosomes prepared using the molar ratio Sp 40: Ch (2:1) show an endothermic peak at  $44.26^\circ\text{C}$ . A DSC thermogram of silymarin loaded niosomes of the same molar ratio show disappearance of the melting endotherm of silymarin and shifting of the endothermic peak at  $35.26^\circ\text{C}$ . The absence of the melting endotherm of silymarin and shifting and/or broadening of the endotherms of surfactant bilayers of niosomes suggest possible interaction of silymarin with bilayer components and can account for the enhanced entrapment of silymarin into these formulations<sup>47-48</sup>.

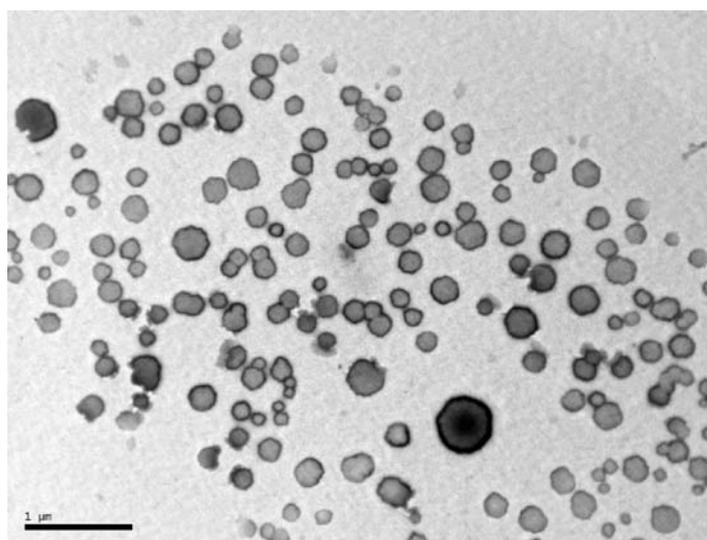


Fig. 5: Electron micrograph of positive drug-free niosomal suspensions of the molar ratio Sp40: Ch: SA (1:1:0.1) at magnification power of 20,000x

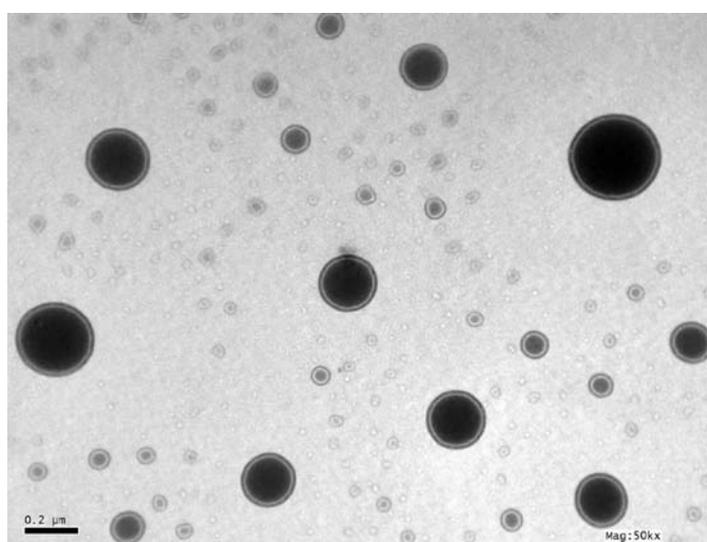


Fig. 6: Electron micrograph of negative silymarin niosomal suspensions of the molar ratio Sp40: Ch: DCP (2:1:0.25), Magnification 50,000

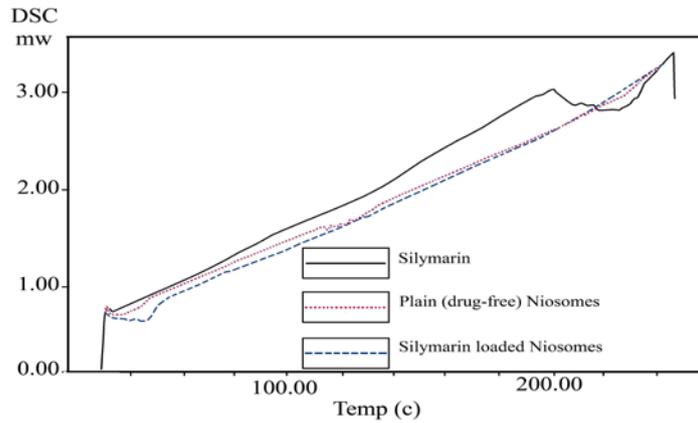


Fig. 7: DSC thermograms of silymarin, plain (drug-free) niosomes and silymarin loaded Sp40 niosomes

**In-vitro release profiles**

The results, tabulated in table (2) and shown in figures (8 and 9), illustrate the amount of silymarin retained in span 60 and span 40 niosomes, respectively, after the periods of time investigated. The results reveal that the release of silymarin from either span 60 or span 40 niosomes was biphasic, with an initial faster release followed by a period of slow release. This biphasic release pattern seems to be a characteristic of bilayered vesicles. Similar results were reported in case of liposomes<sup>49</sup> and in case of niosomes<sup>50-51</sup>. Rapid drug leakage was observed during the initial phase where about 15–35% of the entrapped drug was released from various formulations in the first six hours. However, during the following 66 hrs slow release occurred in which only further 6–10% of silymarin was released from different niosomal preparations.

This could be explained by that the drug is mainly incorporated between the bilayers of niosomal vesicles which lead to rapid release upon dispersing niosomes in buffer until reaching equilibrium<sup>50</sup>.

**Effect of cholesterol content on silymarin release from span 60 and span 40 niosomes**

Considering Span 60 niosomes, Table 2 reveals that the increase in cholesterol content from 33% in neutral niosomes of the molar ratio Sp 60: Ch (2:1) to 50% in the molar ratio Sp 60: Ch (1:1) significantly increased ( $P < 0.05$ ) the percentages of silymarin retained inside niosomes at all times investigated. Upon comparing the two molar ratios Span60: Cholesterol: DCP (2:1:0.25) and Span60: Cholesterol: DCP (1:1:0.1) the same could be noticed.

Table 2: In-vitro release profiles of silymarin loaded Span 60 and Span 40 niosomes of different charges and molar ratio

Time (hr)	Mean Silymarin Retained (%) ± S.D.							
	Neutral Sp 60: Ch		Negative Sp 60: Ch: DCP		Neutral Sp 40: Ch		Negative Sp 40: Ch: DCP	
	1:1	2:1	1:1:0.1	2:1:0.25	1:1	2:1	1:1:0.1	2:1:0.25
Zero	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
3	90.9 ± 1.71	79.68 ± 0.44	92.36 ± 1.07	83.8 ± 2.01	92.18 ± 2.401	83.44 ± 0.500	82.29 ± 1.947	92.85 ± 0.714
6	79.98 ± 1.93	71.12 ± 0.37	80.63 ± 1.14	66.3 ± 1.64	86.64 ± 1.734	75.15 ± 0.241	76.17 ± 1.798	80.15 ± 2.791
24	77.99 ± 2.06	68.13 ± 0.63	78.40 ± 1.40	63.12 ± 1.77	79.07 ± 2.17	70.35 ± 1.344	71.83 ± 2.058	75.94 ± 2.531
48	72.46 ± 2.73	66.49 ± 0.63	73.63 ± 1.02	61.76 ± 2.21	77.16 ± 1.86	68.54 ± 1.010	69.65 ± 1.613	75.04 ± 2.475
72	70.86 ± 2.64	62.33 ± 1.37	71.69 ± 0.92	60.22 ± 2.07	75.92 ± 1.73	67.70 ± 1.010	68.46 ± 1.474	74.50 ± 2.336

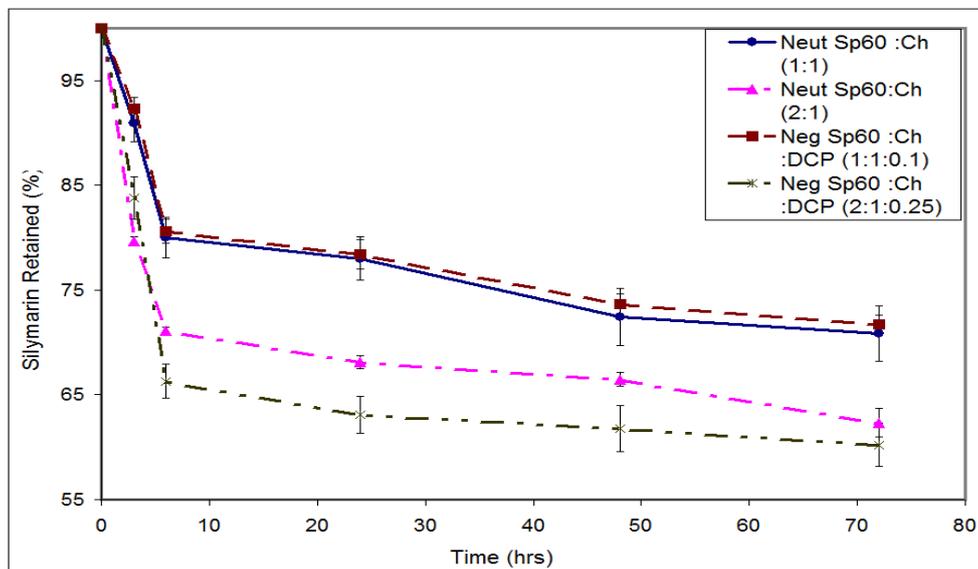


Fig. 8: In-vitro release profiles of silymarin loaded Span 60 niosomes of different charges and molar ratio

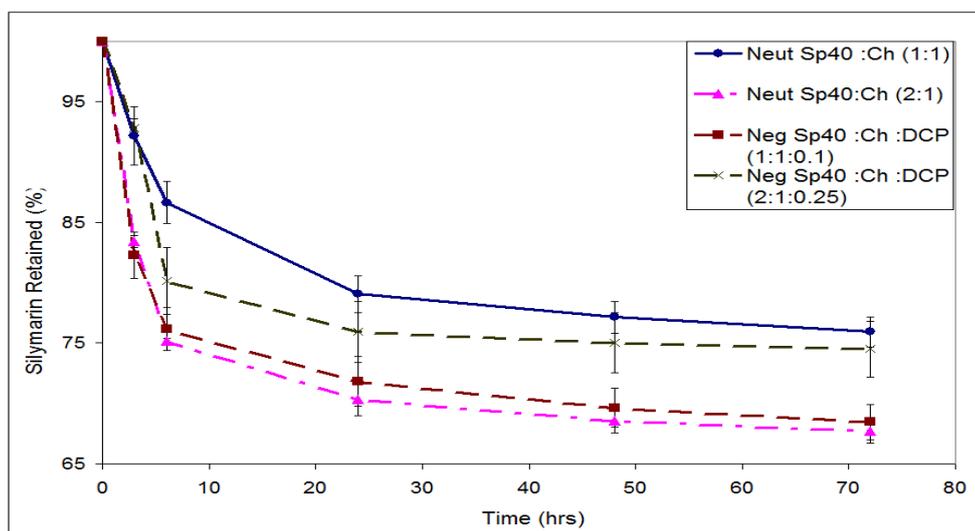


Fig. 9: *In-vitro* release profiles of silymarin loaded Span 40 niosomes of different charges and molar ratios

The increase in cholesterol content significantly increased the percentage of silymarin retained inside niosomes from  $66.3 \pm 1.64$  to  $80.63 \pm 1.14$  and from  $63.12 \pm 1.77$  to  $78.40 \pm 1.40$  after

6 hours ( $P=0.005$ ) and 24 hours ( $P=0.001$ ), respectively. Investigating span 40 niosomes, the results (table 2) reveal that increasing cholesterol content from 33% in neutral niosomes of the molar ratio Sp60: Ch (2:1) to 50% in neutral niosomes of the molar ratio Sp 60: Ch (1:1) significantly increased ( $P=0.045$ ) the drug retained percentage from  $75.15 \pm 0.241$  to  $86.64 \pm 1.734$  at  $t=6$  hours. These results are in accordance with cholesterol membrane stabilizing ability and space filling action<sup>3, 52</sup>. Cholesterol is known to increase the rigidity of niosomes and abolish the gel-to-liquid phase transition of niosomal systems resulting in niosomes that are less leaky<sup>53</sup>, thus decreasing the drug release from niosomes<sup>54</sup>. Cholesterol also alters the fluidity of chains in bilayers and increases the degree of orientation order leading to decreased permeability<sup>55</sup>.

On the other hand, comparing the two molar ratios Sp40: Ch: DCP (2:1:0.25) and Sp40: Ch: DCP (1:1:0.1), the opposite could be noticed (table 2). The increase in cholesterol content did not lead to an increase in silymarin retained percentage. This maybe attributed to the effect of charge inducer, where in this case the effect of charge overcame that of cholesterol content. This confirms that DCP stabilizes the structure of niosomes and renders it less permeable<sup>56</sup>.

#### Effect of charge inducing agents on silymarin release from span 60 and span 40 niosomes

Table 2 show that the difference between the amount of drug retained inside neutral niosomes, prepared using the molar ratio Sp 60: Ch (1:1), and that from negative niosomes, of the molar ratio Sp60: Ch: DCP (1:1:0.1), appeared to be insignificant ( $P>0.05$ ) at all times investigated. Upon comparing the amount of silymarin retained inside neutral niosomes of the molar ratio Sp60: Ch (2:1) and that in negatively charged niosomes of the molar ratio Sp60: Ch: DCP (2:1:0.25), it can be concluded that the inclusion of DCP significantly increased ( $P<0.05$ ) the amount of silymarin retained at all times investigated. Same was noticed on comparing the release extent of

drug from neutral niosomes of the molar ratio Sp40: Ch (2:1) and that from negatively charged niosomes of the molar ratio Sp40: Ch: DCP (2:1:0.25), where the inclusion of DCP significantly increased ( $P<0.05$ ) drug retained percentage at all times investigated. This comes in accordance with the effect of DCP in stabilizing the niosomal membrane structure, rendering it less permeable<sup>56</sup>.

#### Particle size and distribution of niosomal vesicles

Particle size distribution of span 60 and span 40 niosomes are illustrated in table 3 and table 4, respectively. Particle size distribution of silymarin niosomes appeared to be bimodal with the 1st peaks showing the highest contribution.

#### Effect of charge inducers on particle size diameter

The results illustrated in Table 3 reveal that charged niosomes exhibited mean particle diameter higher than that of the neutral ones, viz  $491.2 \pm 68.0$  nm and  $541.1 \pm 73.3$  for negatively and positively charged niosomes of the molar ratio Sp 60: Ch : CIA (1:1:0.1) compared to  $319 \pm 36.2$  nm for neutral silymarin niosomes of the same molar ratio. This could also be noticed upon comparing the mean particle diameter of charged niosomes of the molar ratios Sp 60: Ch: CIA (2:1:0.25) to that of neutral niosomes of the molar ratio Sp 60: Ch (2:1), where charged niosomes exhibited mean particle diameter higher than that of the neutral ones, viz  $332.6 \pm 47.3$ nm and  $397.6 \pm 42.5$  nm for negatively and positively charged niosomes, respectively, of the molar ratio Sp 60: Ch: CIA (2:1:0.25) compared to  $256.2 \pm 29$  nm for neutral silymarin niosomes of the molar ratio Sp 60: Ch(2:1). The results illustrated in Table. 4 also, show that negatively charged niosomes of the molar ratio Sp 40: Ch: DCP (2:1:0.25) exhibited higher mean particle diameter than that of the neutral niosomes of the molar ratio Sp 40: Ch (2:1), viz  $277.2 \pm 43.8$  nm for negatively charged niosomes of the molar ratio Sp 40: Ch: DCP (2:1:0.25) compared to  $219.0 \pm 32.5$  nm for neutral silymarin niosomes of the molar ratio Sp 40 : Ch (2:1). These results could be attributed to that the inclusion of a charge inducer in niosomes leads to an increase in the spacing between adjacent bilayers<sup>57-58</sup>.

Table 3: Particle size distributions of silymarin span 60 niosomes

Formulation	Particle diameter (nm) $\pm$ S.D. (% Area of the peak indicating the volume of particle population)	
	1 <sup>st</sup> Peak	2 <sup>nd</sup> Peak
	Sp 60: Ch (1:1)	$319 \pm 36.2$ (74.0%)
Sp 60: Ch: DCP (1:1:0.1)	$491.2 \pm 68.0$ (94.8%)	$8733.3 \pm 1259.3$ (5.2%)
Sp 60: Ch: SA (1:1:0.1)	$541.1 \pm 73.3$ (95.1%)	$8803.0 \pm 658.6$ (4.9%)
Sp 60: Ch (2:1)	$256.2 \pm 29$ (87.9%)	$3082.4 \pm 360.5$ (12.1%)
Sp 60: Ch: DCP (2:1:0.25)	$332.6 \pm 47.3$ (89.7%)	$2972.5 \pm 413.0$ (10.3%)
Sp 60: Ch: SA (2:1:0.25)	$397.6 \pm 42.5$ (92.2%)	$8960.3 \pm 578.7$ (7.8)

Table 4: Particle size distributions of silymarin span 40 niosomes

Formulation	Particle diameter (nm) $\pm$ S.D. (% Area of the peak indicating the volume of particle population)	
	1 <sup>st</sup> Peak	2 <sup>nd</sup> Peak
	Sp 40: Ch (1:1)	339 $\pm$ 42.3 (76.8 %)
Sp 40: Ch: DCP (1:1:0.1)	185.1 $\pm$ 31.6 (94.5%)	2010.5 $\pm$ 391.0 (5.5 %)
Sp 40: Ch: SA (1:1:0.1)	234.8 $\pm$ 41.1 (93.0%)	2998.0 $\pm$ 663.4 (6.2%)
Sp 40: Ch (2:1)	219.0 $\pm$ 32.5 (89.0%)	1870.8 $\pm$ 294.9 (11.0%)
Sp 40: Ch: DCP(2:1:0.25)	277.2 $\pm$ 43.8 (93.2%)	2944.1 $\pm$ 387.3 (13.2%)
Sp 40: Ch: SA (2:1:0.25)	157.7 $\pm$ 19.1 (97.9%)	7160.1 $\pm$ 1144.5 (2.1 %)

#### Effect of cholesterol content on particle size diameter

Table 3 show that the increase in cholesterol content from 33% in the molar ratio span60: Cholesterol (2:1) to 50% in the molar ratio Span60: Cholesterol (1:1) led to an increase in the particle size diameter, viz 256.2  $\pm$  29 nm for neutral silymarin niosomes of the molar ratio Span 60: Cholesterol (2:1) compared to 319  $\pm$  36.2 nm for neutral silymarin niosomes of the molar ratio Span 60: Cholesterol (1:1). Same conclusions could be depicted upon comparing the mean particle diameter of charged niosomes of the molar ratio Sp60: Ch: CIA (2:1:0.25) to that of charged niosomes of the molar ratio Sp60: Ch: CIA (1:1:0.1), where increasing the cholesterol content led to an increase in the particle diameter viz 332.6  $\pm$  47.3 nm and 397.6  $\pm$  42.5 nm for negatively and positively charged niosomes of the molar ratio Span 60: Cholesterol: CIA (2:1:0.25) compared to 491.2  $\pm$  68.0 nm and 541.1  $\pm$  73.3 nm for negatively and positively charged niosomes of the molar ratio Span 60: Cholesterol: CIA (1:1:0.1), respectively. Cholesterol increases the width of the bilayers and consequently increases the vesicle size<sup>59</sup>.

Considering Sp 40 niosomes, the results (table 4) reveal that an increase in cholesterol content in neutral niosomes led to an increase in the particle size diameter, viz 219.0  $\pm$  32.5 nm for neutral silymarin niosomes of the molar ratio Sp 40: Ch (2:1) compared to 339  $\pm$  42.3 nm for neutral silymarin niosomes of the molar ratio Sp 40: Ch (1:1). Same conclusion could be observed upon comparing the mean particle diameter of the positively charged niosomes, where increasing the cholesterol content led to an increase in the particle diameter from 157.7  $\pm$  19.1 nm for positively charged niosomes of the molar ratio Sp 40: Ch: SA (2:1:0.25) to 234.8  $\pm$  31.1 nm for positively charged niosomes of the molar ratio Sp 40: Ch:SA(1:1:0.1).

#### In-Vivo Study

The effect of silymarin, silymarin niosomal formulations and plain niosomes on activities of serum SGPT, SGOT and SALP in rats after

induction of liver damage by CCl<sub>4</sub> is tabulated in table 5 and illustrated in figure 10. The in vivo results revealed that the administration of the drug loaded niosomal suspensions N1 and N2 (groups V and VI) did not show any change when compared to control group I.

Acute CCl<sub>4</sub> administration resulted in a significant ( $P < 0.001$ ) increase in SGPT to 55.16  $\pm$  10.53U/L compared to normal value which was 24.04  $\pm$  7.83 U/L. Administration of plain niosomes produced a non significant decrease in serum GPT to 45.56  $\pm$  10.10 U/L ( $P = 0.063$ ). Administration of silymarin suspensions, N1 and N2, produced a significant decrease in SGPT levels ( $P < 0.001$ ) to reach 35.96  $\pm$  9.42, 24.49  $\pm$  5.6 and 25.47  $\pm$  1.54 U/L, respectively. At the same time, both niosomal formulations (N1 and N2) showed a significant decrease in SGPT levels in comparison to silymarin suspension ( $P < 0.05$ ).

Concerning the biochemical parameter SGOT, acute CCl<sub>4</sub> administration resulted in a significant ( $P < 0.001$ ) increase in SGOT to 138.02  $\pm$  17.88 U/L compared to normal value which was 55.12  $\pm$  17.39 U/L. Plain niosomes produced an insignificant change in serum SGOT to reach 123.65  $\pm$  1.40 U/L ( $P = 0.631$ ). Significant decrease in SGOT levels upon administration of silymarin suspension, N1 and N2 to reach 89.11  $\pm$  10.26, 50.06  $\pm$  6.27 and 54.62  $\pm$  8.33 U/L, respectively ( $P < 0.001$ ) was found. At the same time, both silymarin niosomal suspensions, N1 and N2 showed a significant decrease in SGOT levels relative to that of silymarin suspension ( $P < 0.001$ ).

Concerning the SALP analysis results, acute CCl<sub>4</sub> administration resulted in a significant ( $P < 0.001$ ) increase in serum SALP to 666.99  $\pm$  18.87 U/L compared to normal value which was analyzed as 265.1  $\pm$  18.76 U/L. Plain niosomes produced a significant change in serum SALP to reach 381.5  $\pm$  19.25 U/L ( $P < 0.05$ ). Significant decrease in SALP levels upon administration of silymarin suspensions, N1 and N2, to reach 302.4  $\pm$  18.78, 271.2  $\pm$  15.92 and 266.5  $\pm$  7.33 U/L, respectively ( $P < 0.001$ ) was produced. Meanwhile, these suspensions, viz; N1 and N2, showed a significant decrease in SGOT levels relative to that of silymarin suspension ( $P < 0.01$ ).

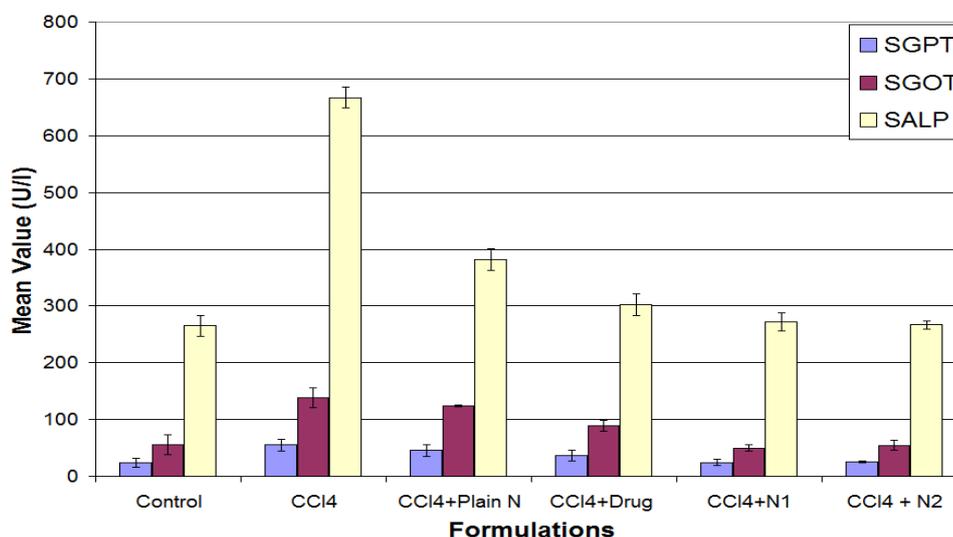


Fig. 10: Effect of silymarin, silymarin niosomal formulations and plain niosomes on activities of serum GPT, GOT and ALP in rats after induction of liver damage by CCl<sub>4</sub>

**Table 5: Effect of silymarin, silymarin niosomal formulations and plain niosomes on activities of serum GPT, GOT and ALP in rats after induction of liver damage by CCl<sub>4</sub>**

Groups	GPT (U/l)	GOT (U/l)	SALP (U/l)
Control	24.04 ± 7.83 (3.19)	55.12 ± 17.39 (7.78)	265.1 ± 18.76 (8.39)
CCl <sub>4</sub>	55.16 ± 10.53 (4.70)*	138.02 ± 17.88 (7.99)*	666.99 ± 18.87 (8.44)*
CCl <sub>4</sub> + Plain N	45.56 ± 10.10 (4.52)*	123.65 ± 1.40 (0.628)*	381.5 ± 19.25 (8.61)* †
CCl <sub>4</sub> + drug	35.96 ± 9.42 (4.12)* †	89.11 ± 10.26 (4.59)* †	302.4 ± 18.78 (8.40)* †
CCl <sub>4</sub> + N1	24.49 ± 5.6 (2.11) † †	50.06 ± 6.27 (2.56) † †	271.2 ± 15.92 (7.12) † †
CCl <sub>4</sub> + N2	25.47 ± 1.54 (0.63) † †	54.62 ± 8.33 (3.40) † †	266.5 ± 7.33 (3.27) † †

Values are mean ± S.D. (S.E.)

Values of \*\* exhibit significant changes from control group, P < 0.05.

Values of † exhibit significant changes when compared to CCl<sub>4</sub> group, P < 0.05.

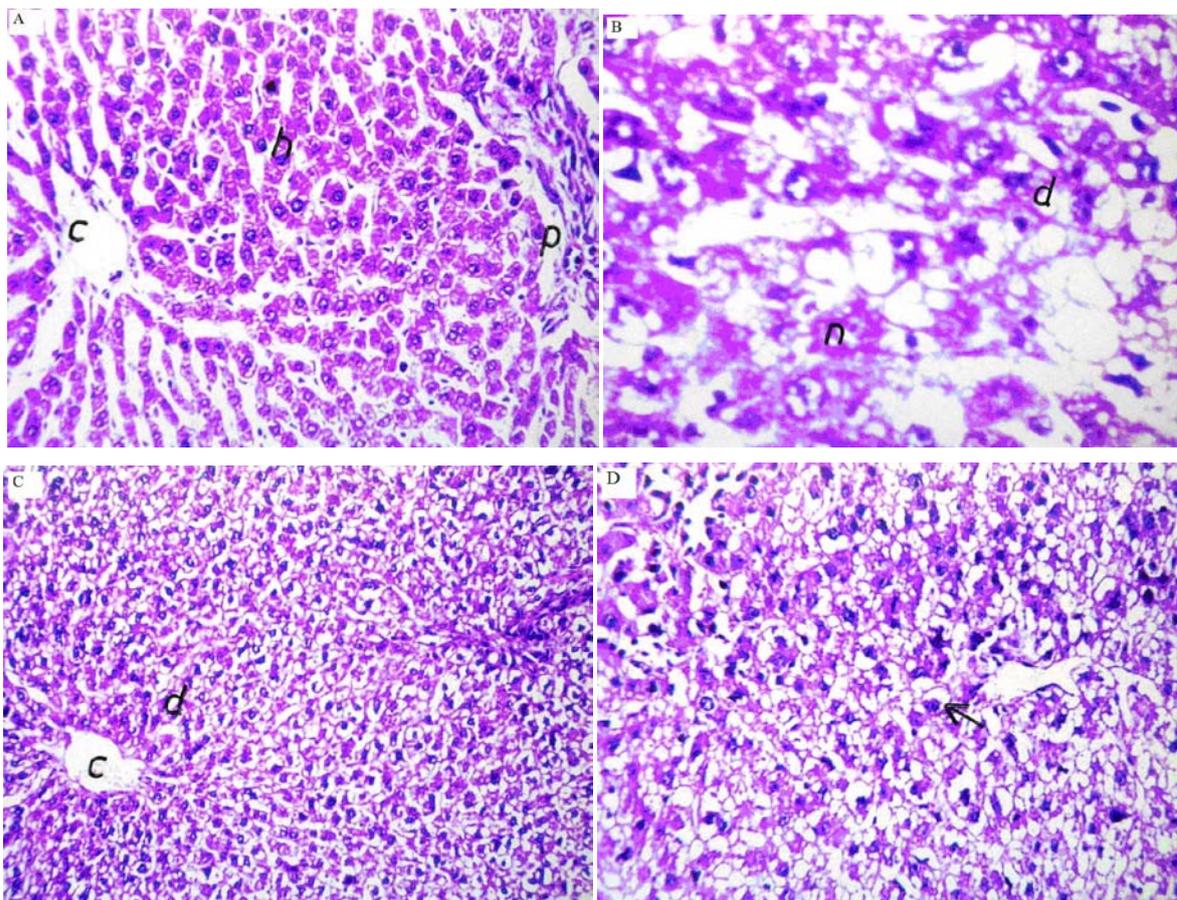
Values of †† exhibit significant changes when compared to (CCl<sub>4</sub> + drug suspension) group, P < 0.05.

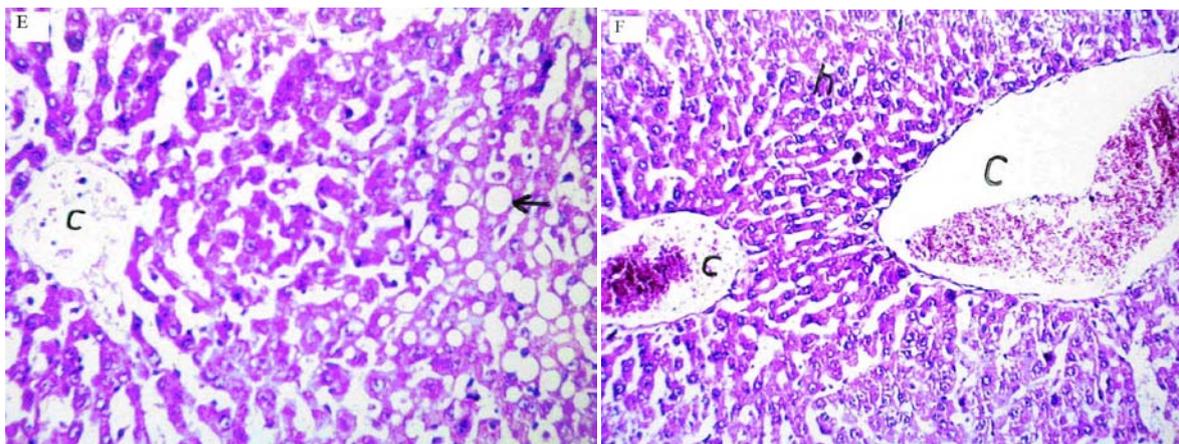
### Histopathological Studies

Histopathological studies (Fig. 11.) show that CCl<sub>4</sub> induced vacuolar degenerative changes and necrosis in the hepatocytes surrounding the central veins and portal area. Administration of plain niosomes also caused degeneration in the hepatocytes all over the hepatic parenchyma. By examination under the microscope, the total area of necrosis and hepatic lesions induced by CCl<sub>4</sub> were reduced by administration of silymarin suspension. Administration of both silymarin niosomal formulations, N1 and N2, showed more improvement, than silymarin suspension in the hepatocytes structure and degenerative areas. Both silymarin loaded niosomal formulations N1 and N2 succeeded to minimize the vacuolar degeneration and necrosis. These results are in accordance with the result of the serum SGPT, SGOT and SALP levels in which administration of N1 and N2 showed better protection against CCl<sub>4</sub> induced damage in comparison with silymarin suspension (table 5 and fig. 10).

### CONCLUSION

This study showed that the niosomal formulation could be one of the promising delivery systems for the hepatoprotective drug silymarin. It provided successful preparation with efficient encapsulation of silymarin. Niosomal formulations characterization using TEM showed the spherical shape and the bilayered structure of the prepared niosomes. Studies using DSC gave evidence of possible interaction of silymarin with bilayer components. In-vitro release profiles were biphasic, with an initial faster release followed by a period of slow release. In-vivo study, performed on rats, proved that silymarin is an efficient hepatoprotective drug and that the investigated niosomal formulations significantly improved the hepatoprotective efficiency. Accordingly, subcutaneous administration of niosomal silymarin formulations is expected to increase drug bioavailability. Drug niosomal formulations were also proved to be safe according to the histopathological investigation.





**Fig. 11: Photomicrographs of histological sections (hematoxylin and eosin stained) representing (a) liver of normal rat (64 x), treated with: (b) CCl<sub>4</sub> (160 x); (c) CCl<sub>4</sub> and plain niosomes (64 x); (d) CCl<sub>4</sub> and silymarin suspension (160 x); (e) CCl<sub>4</sub> and silymarin loaded niosomes N1 (64 x); (f) CCl<sub>4</sub> and silymarin loaded niosomes N2 (64 x), central vein (C); portal area (P); hepatocytes (h); necrosis (n); vacuolar degeneration (d); hydropic degeneration of hepatocytes (reversible) (→)**

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