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Evaluation of hybrid liposomes-encapsulated silymarin regarding physical stability and in vivo performance

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Abstract

Silymarin, a known standardized extract obtained from seeds of *Silybum marianum* is used in treatment of liver diseases of varying origins. Aiming at improving its poor bioavailability from oral products, silymarin hybrid liposomes are introduced in this work for buccal administration after investigating their stability and in vivo hepatoprotective efficiency. Silymarin loaded hybrid liposomes composed of lecithin (L), cholesterol (Ch), stearyl amine (SA) and Tween 20 (T20) in molar ratio of (9:1:1:0.5) were prepared. Their stability upon storage was studied at 4 °C and at ambient conditions. Stored samples were analyzed for percent encapsulation, drug release, particle size, turbidity measurement and visual changes. Characterization of the blend between phospholipid and silymarin was done using FT-IR and DSC which indicated a possible interaction. The stabilized formula of silymarin hybrid liposomes was evaluated upon buccal administration regarding its hepatoprtective activity against carbon tetrachloride-induced oxidative stress in albino rats. The degree of protection was measured using biochemical parameters like serum glutamic oxalacetate transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT). The introduced silymarin hybrid liposomes produced a significant decrease in both transaminase levels when challenged with CCl_4 (intraperitonially) in comparison with orally administered silymarin suspension. This improvement was also confirmed histopathologically.

Keywords: Silymarin; Hybrid liposomes; Stability; Hepatoprotective activity

1. Introduction

Liposomes as a drug delivery system can improve the therapeutic activity and safety of drugs, mainly by delivering them to their site of action and by maintaining therapeutic drug levels for prolonged periods of time (Abou El Wafa et al., 2003; Barragan-Montero et al., 2005; Pavelic et al., 2005). Development of stable liposomes is a prerequisite for its exploitation in the delivery of therapeutic molecules (Sivakumar and Panduranga Rao, 2003; Lau et al., 2005). For many years, attempts have been made to improve the stability of liposomes by several methods, such as, preparing more stable bilayers (Cocera et al., 2003), coating their surface with protecting polymers (Dong and Rogers, 1991; Henriksen et al., 1997; Takeuchi et al., 2001; Guo et al., 2003), adding polyethylene glycol (Moribe et al., 1998; Moribe et al., 1999; Sivakumar and Panduranga Rao, 2001; Hatakeyama et al., 2004), preparation of polymerized liposomes (Okada et al., 1995; Chen and Langer, 1997), charge modification and freeze drying (Winden and Crommelin, 1999; Glavas-Dodov et al., 2005; Sweeney et al., 2005). Some of these methods improved the stability and others were not successful. Also, the type of used phospholipids affected the stability. Liposomes prepared from commercially available phospholipids showed lower stability than synthetic or purified phospholipids (Gadras et al., 1999; Kokkona et al., 2000). However, the latter are more expensive and less available.

Milk thistle (*Silybum marianum*) is one of the few herbal drugs whose excellent pharmacological profile readily lends itself to proof of clinical efficacy (Weiss and Fintelmann, 2000). Meanwhile, silymarin is poorly absorbed (20–50%) from the gastrointestinal tract (Blumenthal et al., 2000). That causes the effects of silybin, one of the main active flavonoids commonly found in the dried fruits of silymarin, to be greater after parenteral than oral administration (Carini et al., 1992). Incorporation of silymarin into liposomal dosage form administered buccaly can improve its bioavailability. In this study, our objective is

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to improve the bioavailability of silymarin through its incorporation in a stable liposomal buccal dosage form, using commercially available soybean lecithin. Such dosage form is designed such that it would avoid instability problems which commonly arouse in the gastrointestinal tract (Vemuri and Rhodes, 1995; Kokkona et al., 2000) and provide a reproducible pharmacological effect through improving the poor solubility of silymarin thus providing a superior dosage form (Madaus et al., 1976; Gabetta et al., 1988; Giorgi et al., 1989; Valcavi et al., 1993). Also, the combination of silymarin with lecithin is intended to increase the permeation of silymarin through buccal mucosa thus enhancing its bioavailability (Gabetta et al., 1988; Comoglio et al., 1995). In a previous work by the authors (El-Samaligy et al., 2006), silymarin encapsulated hybrid liposomes were shown to permeate buccal cheek pouch with promising results of enhancing the drug bioavailability. This is explained by high deformability of liposomes containing Tween 20 as edge activator allowing the medicated hybrid liposomes to squeeze through the buccal mucosal cells (Essa et al., 2002). Accordingly, further studies were done to test stability and detect the in vivo pharmacological activity of the introduced formula.

Because silymarin can produce a hepatoprotective activity against tetrachloride-induced oxidative stress in albino rats in a dose-dependent manner (Datta et al., 1999; Rao et al., 2006), such bioassay can provide useful data in evaluating the efficiency of the introduced buccal liposomal formula in comparison to orally administered silymarin suspension.

2. Materials and methods

2.1. Materials

Lecithin Soya powder (L) was kindly provided by EIPICo (Cairo, Egypt). Cholesterol (Ch) was purchased from Sigma Chemical Co. (USA). Stearylamine (SA) was obtained from Fluca Chemical Co. (Germany). Silymarin was kindly supplied by CID Co. (Cairo, Egypt). Tween 20 (T20), methanol, chloroform, diethylether, sodium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate were purchased from El-Nasr Chemical Co. (Cairo, Egypt). Spectra/Pore® dialysis membrane (12,000–14,000 molecular weight cut off) was purchased from Spectrum Laboratories Inc. (USA).

2.2. Preparation of hybrid liposomes

Silymarin hybrid liposomes were prepared using the reverse evaporation technique (Suzoka and Papahadjopoulos, 1978). The liposomal components were weighed and dissolved in 10 ml chloroform. The organic solvent was evaporated using a rotary evaporator (Buchi R-110 Rotavapor, Switzerland) to produce a thin film. The film was redissolved in 10 ml ether. Silymarin solution in 10 ml acetone together with distilled water was added. The organic solvents were evaporated using rotary evaporator. The liposomal suspension was kept overnight in the refrigerator. On the following day, the liposomal suspension was filtered through sintered glass filter no. 3 and kept in refrigerator. The prepared formula was composed of L:Ch:SA:T20 in the molar ratio of 9:1:1:0.5. The drug recovery was over 95%.

2.3. Determination of silymarin entrapment efficiency in the prepared hybrid liposomes

The proportion of encapsulated silymarin was determined by centrifugating a certain volume of the collected filtrate at 15,000 rpm for 1 h at 4 °C. The liposomes were separated from the supernatant and sonicated with methanol to measure content of encapsulated silymarin at 288 nm, which is the wavelength of maximum absorption of silymarin in methanol (O'Neil et al., 2001):

$$\%E = \frac{\mathrm{ED} \times 100}{\mathrm{AD}}$$

where $\%E$ is the percent encapsulation efficiency, AD the amount of added drug, and ED is the amount of encapsulated drug.

2.4. In vitro release of silymarin from hybrid liposomes

In order to study the silymarin release from liposomes, the dialysis method was applied using a Spectra/Por® dialysis membrane of 12,000–14,000 MWCO (El-Gazayerly and Hikal, 1997). This membrane allows the permeation of the drug and retains liposomal form.

An accurately measured amount (based on drug analysis in liposomal suspension) of silymarin, equivalent to 1 mg silymarin, was transferred to a glass cylinder having the length of 10 cm and diameter of 2.5 cm fitted with presoaked membrane and was placed in a receiving compartment containing 50 ml phosphate buffer saline (pH 7.4). This volume provides complete sink conditions for the drug as the saturated solubility of silymarin is 0.526 mg/ml in this medium. The whole set is placed on a magnetic stirrer adjusted to a constant speed of 150 rpm. at 25 °C. At predetermined time intervals (0.5, 1, 1.5, 2, 3, 4, 5 and 6 h); 4 mL of the release medium were withdrawn for analysis. Withdrawn samples were compensated by phosphate buffer saline (pH 7.4). The samples were measured spectrophotometerically at 326 nm which is the wavelength of maximum absorption of silymarin in phosphate buffer saline (pH 7.4) (Maheshwari et al., 2003).

2.5. In vitro drug absorption and permeation

The chicken cheek pouches (Tan et al., 2000; El-Samaligy et al., 2004) were excised and washed in isotonic buffer (pH 7.4). The open cheek pouch mucosa, with the mucosal surface facing up, was placed between two compartments. The upper compartment contains the liposomal product and the lower one contains isotonic buffer. The exposed surface of the mucosa was 1.23 cm^2. Samples of hybrid liposomal silymarin formulae were applied on the mucosal surface in the donor compartment and receiving compartment was 50 ml of isotonic buffer. Sink conditions were assured by frequently taking samples which were replaced with isotonic buffer and by continuous stirring on a magnetic stirrer

at 150 rpm. Aliquots (4 ml) were removed from the receiving compartment every 0.5, 1, 1.5, 2, 3, 4, 5 and 6 h. The samples were measured spectrophotometerically at $\lambda = 326$ nm.

2.6. Turbidity measurement

Turbidity measurement was carried out to determine the stability of the prepared liposomes against added methanol (Sivakumar and Panduranga Rao, 2003). The liposomes were diluted with distilled water (1:20). An increasing concentration of methanol (0–30%, v/v) was added to the diluted liposomal preparations, mixed well and kept aside at room temperature for 1 h. The absorbance was measured at $\lambda = 400$ nm using an UV–vis spectrophotometer. The measured absorbance was corrected for volume change.

$$\text{corrected absorbance} = \frac{\text{observed absorbance (volume of aqueous vehicle solution + volume of methanol added)}}{\text{volume of aqueous vehicle solution}}$$

2.7. Evaluation of the stability of the prepared silymarin hybrid liposomes

Samples of the prepared liposomal formula were stored either at 4 °C, i.e. refrigerated or at ambient temperature. At predetermined time intervals of 30, 60, and 90 days, the stored samples were analyzed for change in percent encapsulation (Chimanuka et al., 2002). Particle size analysis using laser diffraction particle size analyzer (Mastersizer X), turbidity measurement, drug release and visual inspection were also carried out.

2.8. Investigation of possible interaction between silymarin and soybean lecithin

Interaction between silymarin, a lipophilic drug, and soybean lecithin (phospholipids) was done by adding silymarin to the phopholipid (1:1 ratio). The mixture was kneaded thoroughly with the least amount of water to obtain a paste. The latter was dried under vacuum at room temperature in presence of phosphorus pentoxide as a dessicating agent. The prepared blend was evaluated for possible interaction via the following tests: (a) differential scanning calorimetry (DSC) and (b) Fourier-transform infrared spectroscopy (FT-IR).

2.9. In vivo study on silymarin liposomes

2.9.1. Experimental animals

The study was carried out on male albino rats weighing 180–220 g. The animals were housed in clean cages and maintained in controlled temperature (23 ± 2 °C) and light cycle (12 h light and 12 h dark). They were fed with standard diet and water.

2.9.2. Hepatoprotective activity testing

Animals were divided into five groups each of five rats. Group I was kept as a control group. Rats of remaining four groups received 0.25 ml of carbon tetrachloride (CCl_4) in liquid paraffin (1:1, v/v) per 100 g body weight intraperitoneally (i.p.) to induce hepatic damage (Jose and Kuttan, 2000). Group II did not receive silymarin i.e. acted as untreated group. Groups III, IV and V received silymarin suspension (12.5 mg/kg, p.o.), plain hybrid liposomes and the introduced stabilized medicated hybrid liposomes (12.5 mg/Kg, buccaly), respectively every day (Petkowicz et al., 1990). Silymarin suspension, plain hybrid liposomes and stablilized silymarin hybrid liposomes administration was started 3 days prior to CCl_4 injection and continued till the end of the experiment. After 48 h, animals were sacrificed. Blood was collected and the serum was separated. Collected serum was biochemically tested for transaminase levels of both types SGOT and SGPT. The liver was immediately removed and fixed in 10% formalin, serially sectioned and microscopically examined after staining with hematoxylin and eosin to analyze any pathological changes.

2.10. Statistical analysis

Results of investigating the stored samples and the in vivo study were evaluated using one-way ANOVA followed by 5% allowance test or independent samples *t*-test using SPSS® software. Difference at $P < 0.05$ were considered to be significant.

3. Results and discussion

The prepared silymarin hybrid liposomes formed of L:Ch:SA:T20 at 9:1:1:0.5 molar ratio showed promising drug encapsulation efficiency of 69.22 ± 0.6%.

Stability of the prepared silymarin hybrid liposomes upon storage showed production of aggregates that reflect physical instability of the formula (Fatouros et al., 2001). It was noticed that plane liposomes prepared with the same procedure without adding the drug (unloaded) did not show any physical change. It should be noticed that this behavior was also shown in conventional liposomes encapsulating silymarin. It is possible that encapsulation of silymarin in the liposomal vesicles has a significant effect on their physical properties (Fatouros and Antimisiaris, 2001). Accordingly, to improve the physical stability of the prepared silymarin hybrid liposomes they were mixed with different proportions of unloaded hybrid liposomes composed of L:Ch:SA:T20 of 9:1:1:0.5 molar ratio (same composition and method of preparation as the drug loaded ones). Visual inspection for 3 months showed improvement in the stability of mixtures having 50% or more of the unloaded hybrid liposomes. That may be explained by the fact that unloaded liposomes presence prevents the attraction forces between loaded liposomes that causes fusion and aggregation of the vesicles (Wu et al., 1981). Hence, mixture containing 50% of the unloaded hybrid liposomes (M50) was chosen for further stability testing.

Turbidity measurement was used to calculate the slope of the relation between percent methanol (v/v) added and absorbance at $\lambda = 400$ nm (Cho et al., 1995). It should be noticed that presence of turbidity in presence of 30% methanol informs us that

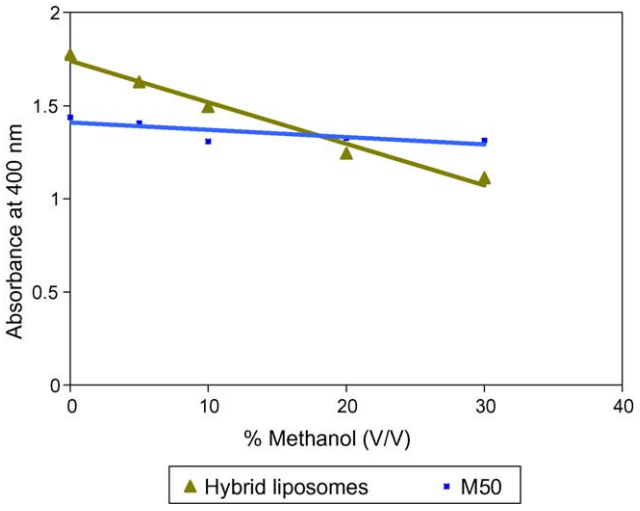

Fig. 1. Relation between % methanol (v/v) added and absorbance at $\lambda = 400$ nm ($n = 3$).

the liposomes are still intact at this alcoholic concentration (Maurer et al., 2001). M50 was compared to hybrid liposomal formula in which turbidity measurement indicated a decrease in the slope of the relation between % methanol added (v/v) and absorbance from −2.2303 to −0.3885 due to increase number of liposomes/ml, Fig. 1. At the same time, M50 did not show any significant change in vitro extent of release after 5 h ($P = 0.211$), Fig. 2, or in silymarin permeation extent through chicken cheek pouch after 6 h ($P = 0.513$), Fig. 3.

Furthermore, M50 was subjected to the stated stability conditions. Analysis of particle size using laser diffraction particle size analyzer showed stability over the stored period at the specified conditions. Liposomes showed a stable average diameter volume

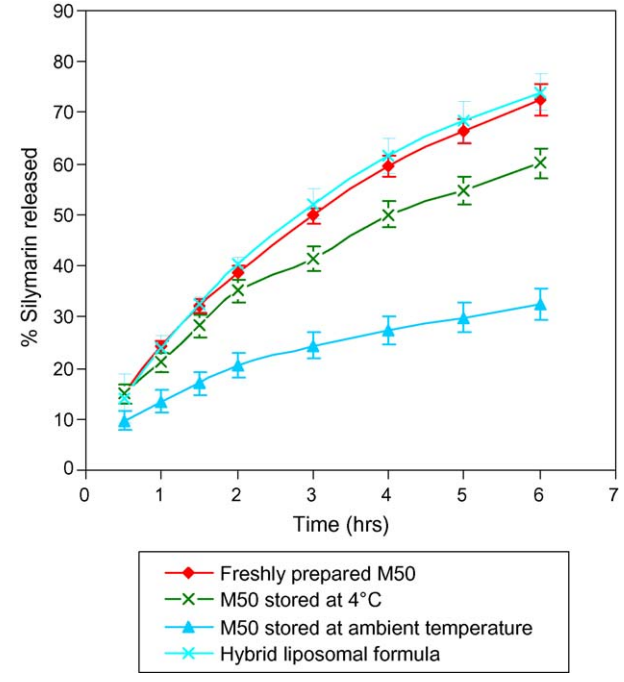

Fig. 2. Release profile of silymarin hybrid liposomes, freshly prepared M50, M50 after 1 month of storage at 4 °C or at ambient temperature ($n = 3$).

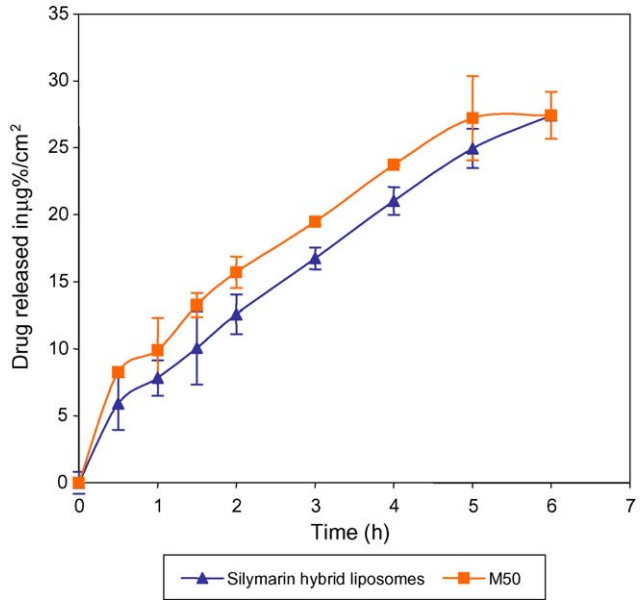

Fig. 3. Permeation profile of silymarin through chicken cheek pouch from hybrid liposomes and M50.

(Du Plessis et al., 1996) of 0.66 µm, Fig. 4. Meanwhile, percent encapsulation efficiency showed a non-significant change over the storage period under the specified conditions. Storage at 4 °C showed a significant decrease in release extent after 5 h when stored for 1 month ($P = 0.024$), Fig. 2. Further storage at this temperature did not produce a significant change in the release extent when compared with the samples stored for 1 month at same temperature ($P = 0.519$ and 0.152 for 2 and 3 months, respectively),

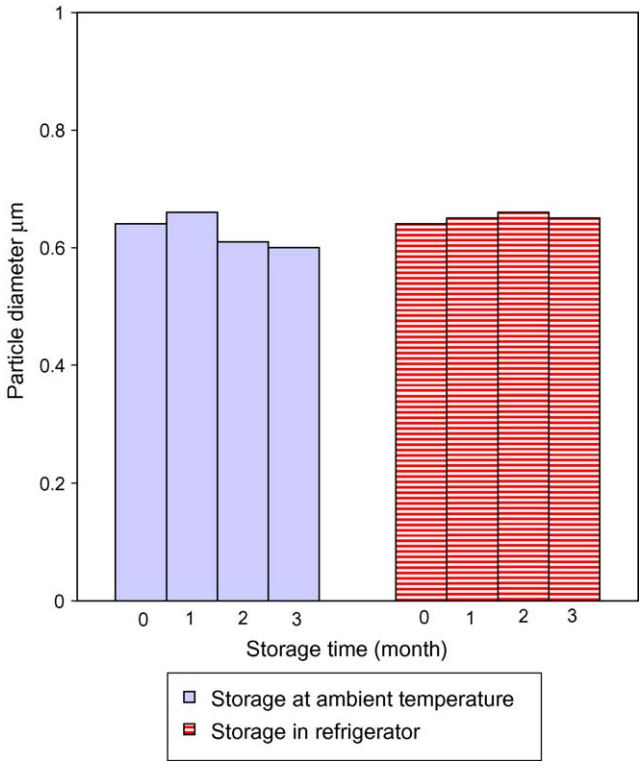

Fig. 4. Effect of storage on liposomal diameters.

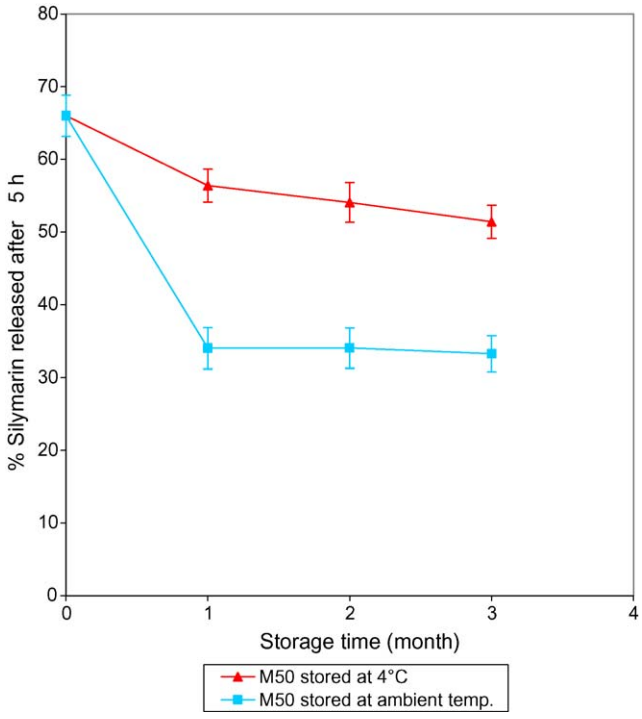

Fig. 5. Percent silymarin released after 5 h for M50 stored at 4 °C and at ambient temperature ($n = 3$).

Fig. 5. Storage at ambient temperature showed a significant decrease in the release extent after 5 h when stored for one month ($P < 0.001$), Fig. 2. Meanwhile, further changes in the release extent after the first month at ambient temperature showed a non-significant change when compared with that of the samples stored for 1 month at same temperature ($P = 1.000$ and 0.742 for 2 and 3 months, respectively), Fig. 5. Observing the decrease in extent of release upon storage for 1 month at 4 °C without any significant change upon further storage can be attributed to complexation between silymarin and the phospholipid that reached its limit after the first month of storage. On the other hand, storage for 1 month at ambient temperature showed more significant decrease in the release extent and that can be caused by presence of other complexation mechanism between silymarin and phospholipid appearing at this temperature since phospholipid present multiple binding sites due to its amphiphilic nature (Abdiche and Myszka, 2004). This complexation also reached its maximum limit after the first month of storage at ambient temperature as no further significant decrease in release extent was observed. The complexation mechanism is one of the interaction mechanisms of the drug with the lipid membrane that represent one of the possible encapsulation mechanisms of the drug into the liposomal vesicles (Moribe et al., 2000). Complex formation between the drug and the phospholipids was investigated by Gabetta et al. (1988) who found that a complex is formed between phospholipids and flavonoids. Similar complexes are also investigated by Moribe et al. (2000), which are known to have higher bioavailability (Comoglio et al., 1995).

Turbidity measurement of M50 showed a non-significant change in the slope over the stored period under the specified conditions ($P = 0.491$). Meanwhile, the UV absorbance

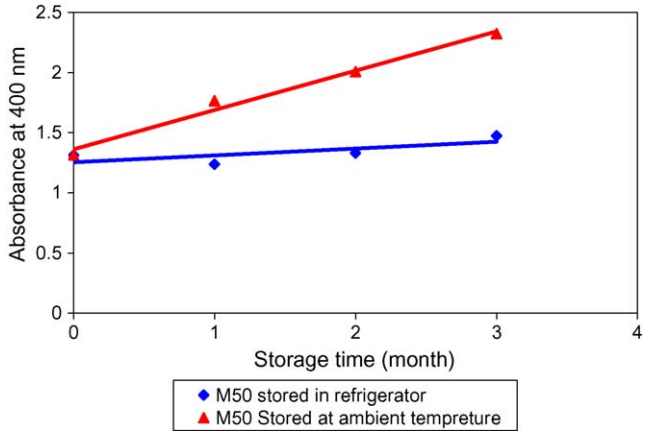

Fig. 6. Turbidity measurement for the stored M50 after adding 30% methanol ($n = 3$).

increased upon storage at ambient temperature (Fig. 6). This increase cannot be attributed to aggregation and fusion mechanism as the laser diffraction particle size analyzer did not reveal change in the particle size frequency distribution curves. This increase could be a result of change in the internal arrangement of molecules inside the liposomal vesicles at ambient temperature which affects UV absorbance. This rearrangement could be self-association or aggregate formation of silymarin in the liposomal membrane (Moribe et al., 2000). These results are in agreement with what has been previously recommended that liposomal suspensions should be kept refrigerated to achieve the best stability as increasing storage temperature contribute to instability in the systems (Du Plessis et al., 1996).

The DSC thermograms of silymarin, the phospholipid and the prepared blend are illustrated in Fig. 7 while their FT-IR are shown in Fig. 8. The disappearance of the drug endothermic peak was observed in the DSC thermogram of the blend. The charac-

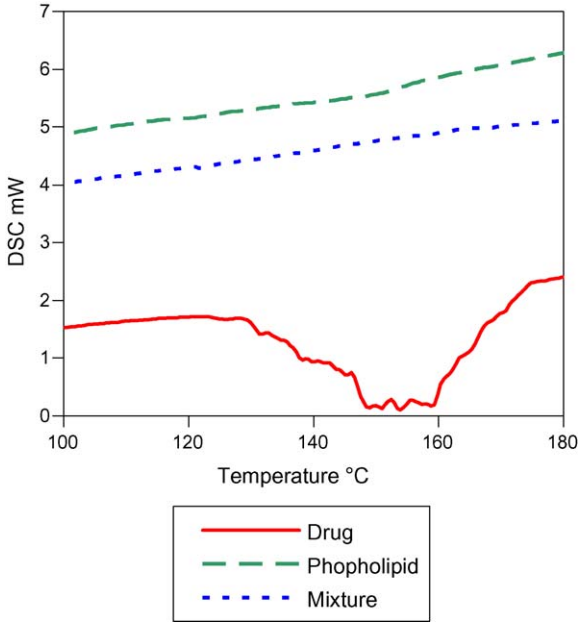

Fig. 7. DSC thermograms of silymarin, phospholipid and the blend.

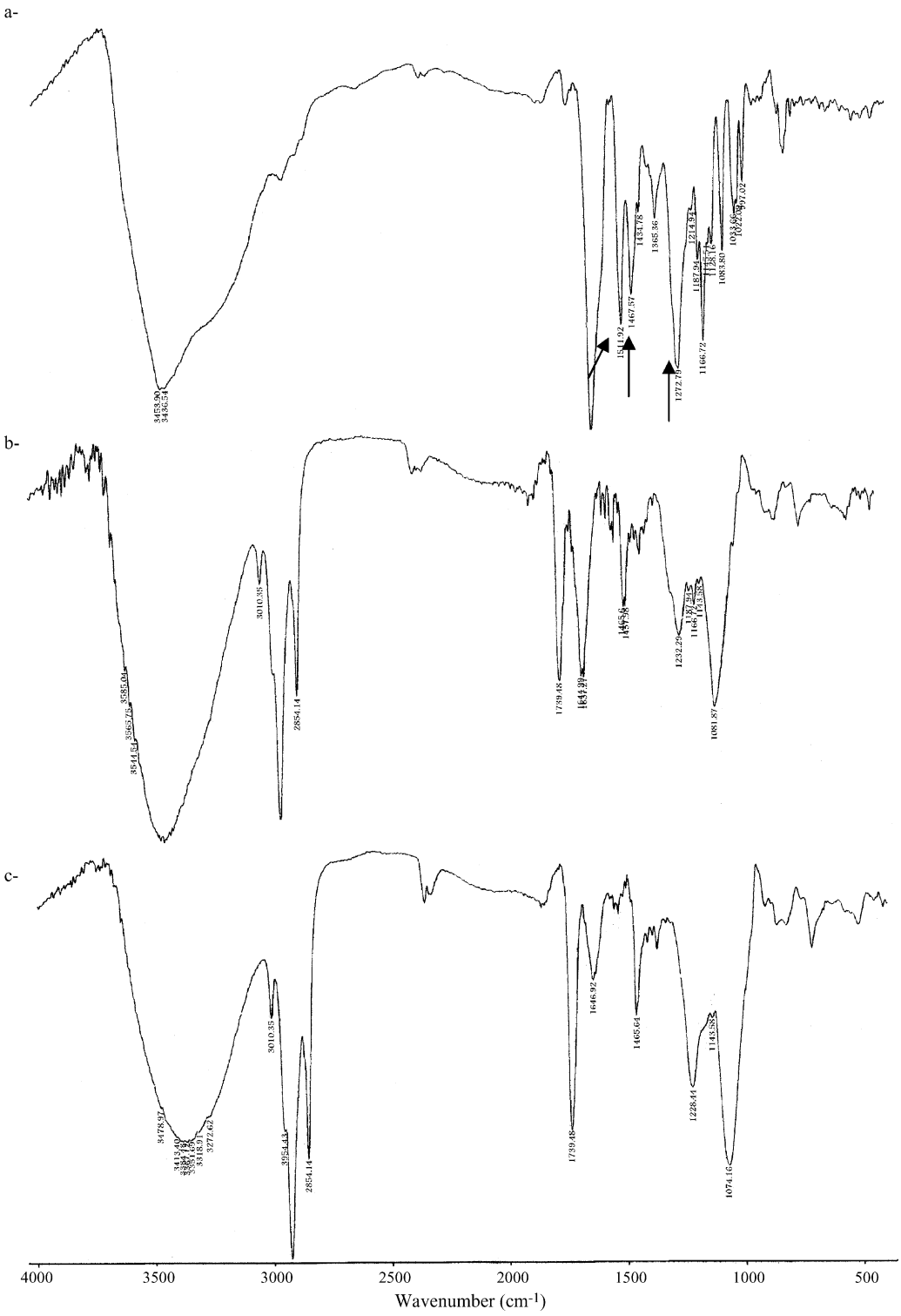

Fig. 8. FT-infrared spectra of silymarin (a), the blend (b) and phospholipid (c).

teristic infrared bands of the drug were markedly affected in the blend; some bands completely disappeared. These changes can provide an evidence of interaction between the lipophilic drug and phospholipid. The interaction may be with the non-polar tails (Immordino et al., 2002). Such interaction may enhance drug permeability which contribute to higher drug bioavailability (Yanyu et al., 2006).

M50 was considered as a stable silymarin loaded hybrid liposomes and thus it was chosen for in vivo study. The in vivo study showed that the buccal mucosa of the rats in group V did not show any change when compared to control group I. Acute CCl_4 administration resulted in a significant ($P < 0.001$) increase in serum SGPT to 1540.20 ± 29.08 U/L compared to normal value which was 29.20 ± 2.16 U/L. Administration of

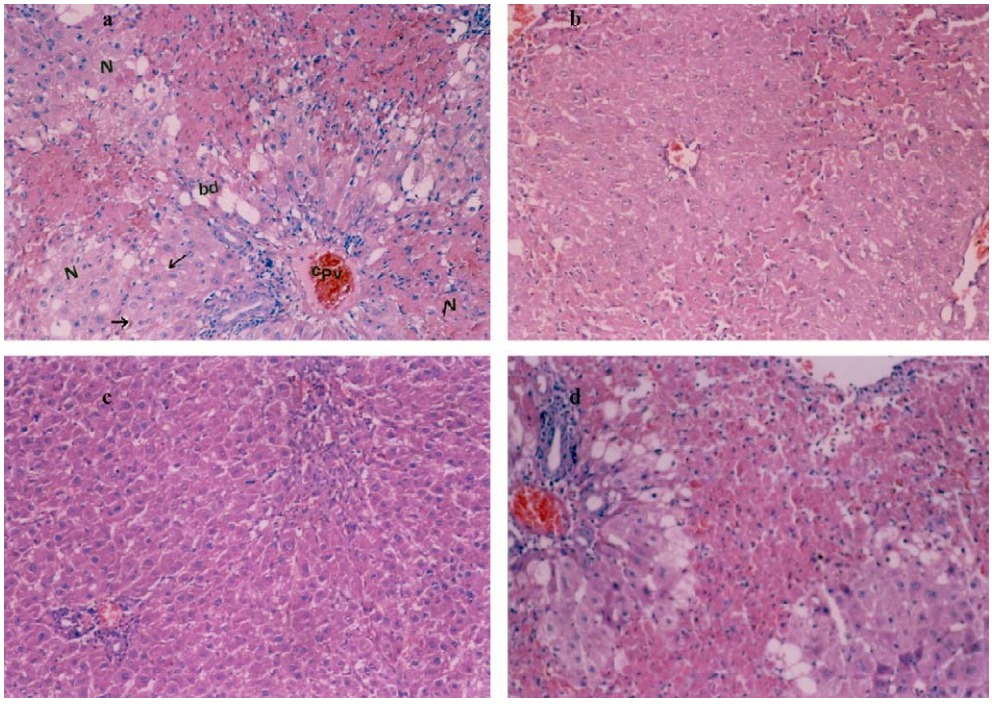

Fig. 9. Photomicrograph (150×) of histological sections (hematoxylin and eosin stained) representing liver of rat treated with: (a) CCl_4; (b) CCl_4 and received oral silymarin suspension; (c) CCl_4 and buccal silymarin hybrid liposomes; (d) CCl_4 and plain hybrid liposomes [necrosis (N); ballooning (bd); vacuolar degeneration of hepatocytes (→)].

plain hybrid liposomes produced a non significant decrease in serum GPT to 1498.20 ± 64.21 U/L ($P = 0.283$). Administration of silymarin suspension and M50 produced a significant decrease in SGPT levels ($P < 0.001$) to reach 802.20 ± 84.65 and 657.60 ± 77.27 U/L, respectively. Meanwhile, M50 showed a significant decrease in SGPT levels in comparison to silymarin suspension ($P < 0.001$).

At the same time, acute CCl_4 administration resulted in a significant ($P < 0.001$) increase in serum SGOT to 2093.00 ± 37.36 U/L compared to normal value which was analyzed as 104.00 ± 3.08 U/L. Plain hybrid liposomes produced non significant change in serum SGOT to reach 2131.60 ± 125.10 U/L ($P = 0.631$). Significant decrease in SGOT levels upon administration of silymarin suspension and M50 to reach 1481.80 ± 200.37 and 1071.60 ± 145.47 U/L, respectively ($P < 0.001$) was produced. Meanwhile, M50 showed a significant decrease in SGOT levels relative to that of silymarin suspension ($P < 0.001$).

Histopathological studies (Fig. 9) showed that CCl_4 induced massive necrosis, hepatic lesions, ballooning, and vacuolar degeneration of hepatocytes. It also revealed congestion in the portal vessels (cpv) with increase cellular infiltration around them. Administration of plain hybrid liposomes also revealed the severe damage observed upon CCl_4 administration in which focal necrosis, ballooning, vacuolar degeneration as well as dilatation and congestion in portal vessels are still present. By microscopic examination, the massive area of necrosis and hepatic lesions induced by CCl_4 were remarkably reduced by administration of oral silymarin suspension. Buccal administration of M50 showed better improvement, than oral silymarin suspension, in the hepatocytes structure, necrotic areas and ballooning damage as demonstrated by comparing Fig. 9b and c. M50 minimized the vacuolar degeneration and liver tissue restored its normal structure. These results are in good agreement with the result of the serum SGPT and SGOT levels in which buccal administration of M50 produced better protection against CCl_4 induced damage in comparison with oral silymarin suspension.

4. Conclusion

This study on silymarin encapsulated hybrid liposomes revealed successful preparation with efficient encapsulation of silymarin. Mixing silymarin loaded hybrid liposomes with unloaded ones in a (1:1) proportion was useful in prevention of aggregates which threaten liposomal stability. M50 proved stability regarding encapsulation efficiency, turbidity measurement and particle size analysis after 3 months of storage at 4 °C or at ambient temperature. Refrigeration is recommended to achieve better stability. Studies using DSC and FT-IR give evidence of possible interaction between silymarin and phospholipids. This may contribute to enhancing bioavailability by increasing drug permeation.

In vivo study documents that silymarin is efficient hepatoprotective drug and that the introduced formula significantly improved the hepatoprotective efficiency. Accordingly, buccal administration of the introduced hybrid liposomal silymarin formula is expected to increase drug bioavailability significantly. The introduced hybrid liposomal silymarin formula for buccal administration have the advantages of exerting a mucoadhesive effect (Takeuchi et al., 2003) beside its deformability due to the

presence of Tween 20 as edge activator allowing the medicated liposomes to squeeze through buccal mucosal cells. It was also shown to be safe upon contacting the rat buccal mucosa.

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