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Niosomal 5-Flourouracil gel for effective treatment of skin cancer; *In-vitro* and *In-vivo* evaluation

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Abstract

This study was designated to form core-enriched 5-flourouracil (5-FU) niosomes and apply it to skin as a niosomal gel for topical treatment of skin cancer. Different molar ratios of the two surfactants used namely; sorbitan monostearate (Span 60), sorbitan monolaurate (Span 20) to cholesterol were employed, in addition; sodium deoxycholate was used a co-surfactant. The drug was successfully entrapped in niosomes with entrapment efficiency reached up to 67.08 ± 2.53 mg % (w/w). The produced niosomes had particle size below 300 nm, zeta potential values between -15 ± -1.6 and -37.73 ± -2.53 mV and polydispersity index between 0.09 ± 0.06 and 0.20 ± 0.02. Transmission electron microscopy showed the formation of spherical niosomes with closed bilayer structure. Formula N8 had more than two fold increase in amount permeated compared to free drug in *in-vitro* permeation study. The niosomal gel formulae had better permeation parameters compared to formulae containing free drug. Niosomal gel formula composed of sodium carboxymethyle cellulose (Na CMC) had the best permeation parameters among the produced gel formulae. Histopathological studies showed that niosomal 5-FU gel was able to penetrate more readily into deep layers of skin to treat tumor as indicated by the reduction in inflammatory reaction and hemorrhage signs observed in animals treated by niosomal 5-FU gel.

Keywords: 5-Flourouracil, niosomal gel, skin cancer, transdermal permeation, niosomes.

Introduction

Chemotherapy is one of the main therapeutic strategies for treatment of cancer in addition to surgery, radiation and biologic therapies (immunotherapy and hormonal therapy) [1]). Cytotoxic drugs are the most frequently used chemotherapeutic agents for cancer treatment. The main action of cytotoxic drugs is to destroy cells that are rapidly growing and dividing such as cancer cells. Consequently; cancer cells are the most preferentially killed cells by cytotoxic agents [2]. However; treatment failure following administration of these drugs is still encountered and the clinical results are frequently less than desired.

5-Fluorouracil is a hydrophilic anticancer drug that is used in management of many types of skin cancer. A 1% solution of 5-FU in water has a pH of 4.5 to 5.0 [3, 4]. The mechanism of action of 5-FU involves the interference with nucleoside metabolism and incorporation into RNA and DNA, eventually resulting in cell death. Actually; this drug has a reasonable anti-cancer activity against lesions related to squamous cell carcinoma such as Bowen's disease [5].

Due to its hydrophilic and acidic nature, the permeation of 5-FU across the lipophilic layers of the skin is found to be very low. Additionally, 5-FU is metabolized with high rate in the body, so; to maintain a reasonable therapeutic concentration , high doses of the drug will be administered, which could induce great toxic effects [6, 7]. For these reasons; many researchers investigated other techniques to enhance its transdermal permeation. These techniques included; use of permeation enhancers, iontophoresis [8], laser treatment [9], prodrug technique [10] and many others [11]. Development of resistance by cancer cells against 5-FU had decreased the clinical use of 5-FU over the past 50 years. For example; it was reported that the use of 5-FU alone in treating colorectal cancer resulted in only 10 % response [3].

These disadvantages and other toxic side effects of 5-FU could be decreased by specifically delivering such drug into cancer cells and minimizing the exposure of other healthy cells to this agent

Niosomes are known to be vesicular systems formed by the self-assembly of non-ionic surfactants in aqueous media to form closed bilayer structures. Such assembly [12] frequently requires some energy like physical agitation or heating. Those vesicular delivery systems can provide a way to deliver active agents such as 5-FU into the required target site due to their desirable prosperities such as their ability to entrap hydrophilic drugs such as 5-FU into their core and their ability to pass readily through lipophilic membranes and finally their ability to act as penetration enhancers.

The aim of work of this study is to formulate 5-FU in the form of niosomal gel in order to enhance its anticancer activity through improving its transdermal permeation and help its delivery more precisely into required site of action.

Materials & Methods

Materials

5-FU powder was purchased from Merck Co. (Germany). Cholesterol, sorbitan monostearate (Span 60), sorbitan monolaurate (Span 20), sodium deoxycholate, Phosphate Buffer Saline tablets (PBS) and dialysis bags with a molecular weight cut off of 12000kDa were purchased from Sigma Aldrich (USA). Cellulose nitrate papers with pore size 0.2 µm were purchased from Seitz Co. (Germany). Absolute ethanol, chloroform and Sörensen's Phosphat Buffer, composed of sodium chloride, di-sodium hydrogen orthophosphate and potassium-dihydrogen orthophosphate were purchased from Elgomhoria CO. (Egypt). Sodium carboxymethyle cellulose (Na CMC), hydroxypropylemethyle cellulose (HPMC), chitosan and Hematoxylin and eosin stains were purchased from Sigma Aldrich (USA). All other chemicals and solvents used were of HPLC grade.

Preparation of 5-FU loaded niosomes

5-FU niosomes were prepared with thin film-hydration technique [13]. Briefly; the required quantities of the surfactant (Span20 or Span60) and cholesterol in different molar ratios (1:2 and 1:3) of cholesterol to Span respectively, with or without 0.5 % w/w of the co-surfactant Na deoxycholate (Table 1) were dissolved in 5mL of a solvent mixture (2:1, v/v of chloroform: methanol respectively) in a round-bottom flask. The organic solvents were evaporated using a rotary evaporator (Stuart rotary evaporator (RE300, UK)) at 40◦C for 10 min under vacuum using vacuum pump (Stuart vacuum pump (RE3022C, UK)) to form a thin film on the wall of the flask; the last traces of organic solvents were removed by desiccation under vacuum for 2 h. thin film was hydrated using 10mL of phosphate buffer (pH 7.4) at 55◦C. The resulting niosomal suspension was sonicated in a bath sonicator (Ney ultra sonic cleaner (57 H, USA)) for one hour to obtain multilamellar niosomes.

Table (1): Composition of 5-FU loaded niosomes

Formula number	Drug (mg)	Molar ratio of cholesterol: surfactant	Cholesterol (mg)	Surfactant type	Surfactant concentration (mg)	Na deoxycholate (0.5% w/w)
N1	10	1:2	45	Span60	100	absent
N2	10		45	Span60	100	present
N3	10	1:3	45	Span60	150	absent
N4	10		45	Span60	150	present
N5	10	1:2	55	Span20	100	absent
N6	10		55	Span20	100	present
N7	10	1:3	55	Span20	150	absent
N8	10		55	Span20	150	present

Characterization of 5-FU loaded niosomes

Determination of entrapment efficiency

Drug entrapped into niosomes was separated from free 5-FU by centrifugation of the niosomal suspension at 50000 rpm for one hour (Beckman ultra centrifuge, (GS, 6KR USA)), washed twice and finally recenrifuged to ensure complete removal of the unentrapped 5-FU. The amount of encapsulated 5-FU was determined by lysis of the separated vesicles using 93% ethanol,

heating to 60°C, cooling the solution to room temperature then centrifugation of the solution at 4000 rpm for 30 min. Finally 1ml of the supernatant was diluted to 10 ml using PBS (pH 7.4) and measured spectrophotometrically at λmax 266 nm (Jasco spectrophotometer (V530, USA)).
The 5-FU entrapment efficiency was determined using equation (1)

$$\text{5-FU entrapment efficiency} = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug added}} \times 100 \quad (1)$$

Zeta potential and size measurement

Zeta potential, particle sizes and polydispersity index were determined by diluting 5-FU niosomal suspension by distilled water. The particles size was measured using Malvern PCS4700 system, while zeta potential of nanosuspension samples was determined by using Zetasizer 2000.

Transmission electron microscopy

Morphological characters of 5-FU loaded niosomes were illustrated using transmission electron microscopy (Transmission electron microscope analyzer (Jeol, Japan)). The device was operating at an accelerating voltage of 80 kV. A drop of niosome suspensions was applied onto a cupper grid, stained with urinyle acetate, left to dry and finally examined.

In-vitro diffusion study

Diffusion study was done using different volumes of niosomal suspension carrying fixed weights of 5-FU (2 mg) that were inserted into tube (surface area 5 cm^2) after sealing one its lower surface with cellulose nitrate paper (pore size of 0.2µm) which was immersed in isopropyl myristate overnight to simulate the lipophilic characters of the stratum corneum [14]. The tubes was suspended in PBS (25 ml, pH 7.4) at 37±0.5°C and considered as a receptor chamber. The receptor was stirred at 100 rpm on a magnetic stirrer. Aliquots of one ml were withdrawn from receptor chamber at predetermined time intervals and diluted to 1:10. The receptor chamber was compensated with equal volumes of fresh medium. The withdrawn samples were filtered through 0.45 µm pore filter and finally measured at λmax 266 nm.
The permeation data of 5-FU was graphically plotted as the cumulative amount of drug permeated per unit area as a function of time. The permeation profiles provided permeation parameters namely; the permeability coefficient (Kp) (i.e. the slope of the straight line portion of the curve divided by concentration of drug originally added), lag time (i.e. the X-intercept of the linear portion of the graph) and Q_{24} (i.e. the cumulative amount of the drug permeated per unit area after 24 hours).
Based on the obtained results from the previous tests, formula N8 was chosen to be incorporated in three different gel matrices. Anionic, neutral and cationic Bases namely; Na CMC (3% w/v), (HPMC (2% w/v) and chitosan (1.5%) were used. These matrices were mixed with certain volume of N8 which contained a fixed amount of 5-FU so that the final concentration of drug in the gel was 20 % w/w. They were left to equilibrate in the refrigerator for 24 hours before use. In The diffusion experiment of the gel, about 0.05 mL of the gel containing 2.5 mg of 5-FU was inserted in the donor chamber and the experiment was carried out in a way similar to that used with the niosomes.

Stability study

The stability of vesicles was tested by measuring the encapsulation efficiency and particle size of N8 monthly for 6 months.

In-vivo study

Animals

BALB/c mice with average weight of 18 ± 2 gm were used.

Induction of tumor

The mice were divided into three groups each composed of six animals. Each animal was injected in the right thigh subcutaneously with 0.2 ml 1x 10^6 single cell suspension isolated from Ehrlich ascetics' carcinoma (EAC). The parent line of Ehrlich ascetics' carcinoma was supplied by the National cancer Institute of Cairo University. Animals were housed at the same temperature and humidity and received similar diet regimen. Animals were left for 10 days to allow tumor growth, the hair covering the right thigh was removed using electric hair clipper. Animals were divided into three groups namely; group A that served as negative control (received no drug or external treatment), group B that served as positive control and received free 5-FU topical gel twice daily and group C that Received 5-FU loaded niosome topical gel twice daily. The dose was calculated according to Paget and Barns to be equivalent to that used for humans (i.e. 2.27 mg /ml for animal) [15]. Each animal was put in separate cage and subjected to the treatment with the formula meant for each group. All mice were under observation every day until dead or sacrificed.

Histopathological studies

14 days after tumor implantation part of the mice from group A were sacrificed and biopsies were performed. The tumors that developed at the site of injection of EAC cells were excised and fixed in 10% formaldehyde. The preserved tumor tissue was dehydrated, cleared, and processed for routine paraffin-block preparation using a rotary microtome. Sections of about 5 µm in thickness were cut using rotary microtome (Model 82, American

Optical®, Oregon, USA), stained with hematoxylin, and counter-stained with eosin (H&E) [16]. The slides were examined for Histopathological changes such as inflammatory reaction, necrosis, hemorrhagic areas and hyperkeratosis of the tumor by an observer who was blind with respect to the treatment groups. Tumors from groups B and C were excised just after animals' death, stained using H&E stain and histopathologically examined using light microscope attached with camera (Leica, Germany).

Results & discussion

Niosomes entrapment efficiency

5-FU is a hydrophilic drug so it can be efficiently solubilized in PBS (pH 7.4) and so it can be incorporated in the niosomal core. The obtained results showed that 5-FU has been successfully incorporated into niosomes core by an encapsulation efficiency reached up to 67.08 ± 2.53 % w/w (Table 2). It was obvious that Span 60 containing formulae had higher encapsulation efficiency compared to formulae containing Span 20. This can be attributed to the difference in alkyl chain length of surfactants. The alkyl chain of Span 60 is longer than that of Span 20 and it was reported that the longer the alkyl chain length the lower the permeability of the membrane and the higher the entrapment efficiency [17]. Sodium deoxycholate seemed to decrease the entrapment efficiency as it acts as a co-surfactant and increases the elasticity of the membrane and eventually decreases the entrapment efficiency [18]. It was noticed that increasing Span 60 increased the entrapment efficiency because the higher the amount of Span 60, the lower the permeability of the membrane and the higher the entrapment efficiency. On the contrary, increasing Span 20 decreased the entrapment efficiency as increasing Span 20 will decrease the rigidity of the membrane and finally decrease the entrapment efficiency.

Zeta potential and particle size analysis

Zeta potential values of niosomes ranged from -15 ± -1.6 to -37.73 ± -2.53 mV as can be seen in Table 2. Zeta potential values of Span 20 niosomes were higher than that of Span 60 niosomes this can be ascribed to the hydrophilic nature of Span 20. It was reported in literature that the more hydrophilic the surfactant, the higher the zeta potential values of vesicles [19]. Span 20 has an HLB value of 8.6 which is higher than that of Span 60 that is 4.7. Increasing amount of Span 20 increased zeta potential and the opposite was observed in case of Span 60 niosomes. Nadeoxycholate was noticed to zeta potential values.
drug loaded niosomes had a particle size that ranged from 138 ± 2.3 nm to 274 ± 1.99 nm with polydispersity index ranging from 0.09 ± 0.06 to 0.2 ± 0.02 this indicates that most of the produced niosomes are somewhat monodispersed in the suspension which is a desirable property in such drug delivery system as it enhances stability and lessens the chance of aggregation of the formed particles. Span 60 containing niosomes were larger in size than niosomes containing Span 20. It is well established that the diameter of the vesicles depends on the surfactant's alkyl chain length; consequently surfactants with longer alkyl chains generally give larger vesicles [20]. This could be the reason behind the smaller particle size of span 20 and larger particle size of Span 60 niosomes. Increasing amount of Span 60 increased the particle size, while, increasing Span 20 amount decreased niosomes' size. The co-surfactant Na deoxycholate seemed to decrease particle size. This decrease in particle size could be explained by increased flexibility and reduced surface tension of the vesicles induced by Nadeoxycholate [18].

Table 2: Zeta potential, particle size, polydispersity index and entrapment efficiency values of 5-FU loaded niosomes (mean ± SD)

Formula No.	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment Efficiency (% w/w)
N1	211 ± 3.10	0.20 ± 0.02	-21.00 ± -1.40	52.65 ± 1.03
N2	202 ± 2.00	0.19 ± 0.05	-24.66 ± -1.32	47.73 ± 2.17
N3	274 ± 1.99	0.15 ± 0.03	-15.00 ± -0.99	67.08 ± 2.53
N4	236 ± 3.90	0.16 ± 0.01	-17.00 ± -1.83	60.74 ± 4.00
N5	169 ± 1.74	0.12 ± 0.02	-28.00 ± -1.00	51.06 ± 1.28
N6	164 ± 1.50	0.13 ± 0.04	-31.00 ± -1.97	46.98 ± 1.63
N7	143 ± 1.68	0.09 ± 0.06	-33.66 ± -1.20	40.05 ± 2.04
N8	138 ± 2.30	0.11 ± 0.01	-37.73 ± -0.90	33.56 ± 1.22

Transmission electron microscopy

Transmission electron micrographs illustrates that the formed niosomes were spherical with closed bilayer structure. Figure (1) illustrates the transmission electron micrographs of 5-FU loaded niosomes.

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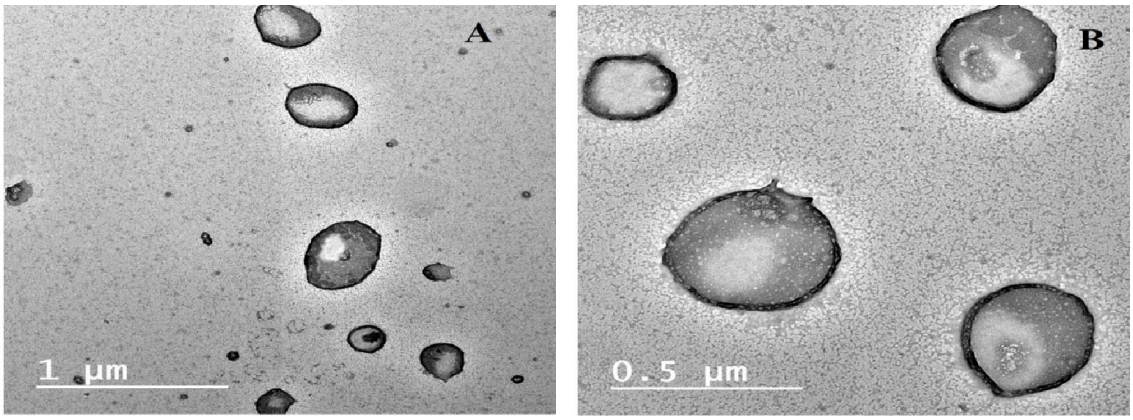

Figure 1: Transmission electron photomicrographs of: A) Span 20 niosomes and B): Span 60 niosomes.

In-vitro diffusion study

From the results listed in Table (3) it's clear that niosomal suspension increased the diffusion of 5-FU from all niosomes formulae compared to free 5-FU. Niosomes are composed of nonionic surfactants, which are biocompatible and relatively nontoxic and themselves serve as excellent penetration enhancers. The niosomes as penetration enhancers may predominantly be on the intercellular lipids of stratum corneum raising the fluidity and weakness of stratum corneum resulting in increased penetration efficacy [21]. Also niosomal formulation as a lipid based drug delivery system may be able to penetrate through the lipophilic membranes like stratum corneum more efficiently than the hydrophilic 5-FU. It was worth noting that 5-FU loaded niosomes containing span 20 permeated more readily than those containing span 60 as a surfactant. This difference can be attributed to smaller particle size of span 20 containing niosomes compared to larger particle size obtained in case of span 60 containing niosomes which in turn will increase the area available for diffusion. Also surfactants of lower Tc like span 20 (Tc =16^{o}C) give more fluid membranes of the vesicles compared to surfactants having higher phase transition temperature like span 60 (Tc = 53^{o}C) and hence allow greater diffusion to take place [20].

Increasing the amount of span 20 resulted in an improvement in diffusion parameters as noticed in formula N8 (composed of 150 mg span 20, 55 mg cholesterol and 0.5% Na deoxycholate) which has permeation parameters considerably better than formula N6 (composed of 100 mg span 20, 55 mg cholesterol and 0.5% Na deoxycholate). In the mean time increasing the concentration of span 60 seemed to decrease the permeation of niosomes. For example formula N1 (composed of 100 mg span 60, 45 mg cholesterol) had better permeation parameters than formula N3 (composed of 150 mg span 60 along with 45 mg cholesterol). Na deoxycholate seemed to contribute to enhancement of 5-FU diffusion from niosomal formulae. In formula N7(containing 150 mg span 20, 55 mg cholesterol but no Na deoxycholate) the permeation parameters were lower than those obtained with formula N8 that has same composition as N7 except that it contains Na deoxycholate. This increment in diffusion parameters in case of presence of Na deoxycholate can be referred to the co-surfactant effect of Na deoxycholate which increases the flexibility of the membrane of niosomes and also gives smaller niosomes with larger surface area and greater area offered for diffusion. Figure (2) illustrated the permeation profiles of 5-FU loaded niosomes along with the plain drug.

Table 3: In vitro permeation parameters of 5-FU from 5-FU loaded niosomes formulae against free 5-FU (mean ± SD).

Formula No.	Q_{24} (ug/cm^2)[a]	Lag time (min)[b]	Kp (cm/hr)[c]
N1	177.0 ± 6.54	50.5 ± 2.98	0.335 ± 0.021
N2	181.3 ± 8.86	46.1 ± 3.10	0.426 ± 0.019
N3	148.0 ± 5.58	59.0 ± 1.99	0.131 ± 0.012
N4	153.5 ± 7.78	54.7 ± 2.00	0.277 ± 0.031
N5	215.0 ± 10.75	49.9 ± 3.20	0.369 ± 0.024
N6	221.0 ± 9.19	38.0 ± 2.70	0.451 ± 0.017
N7	242.0 ± 9.32	32.8 ± 3.50	0.471 ± 0.042
N8	259.0 ± 10.75	25.5 ±3.00	0.611 ± 0.018
Free 5-FU	122.0 ± 4.00	80.0± 3.03	0.065 ± 0.012

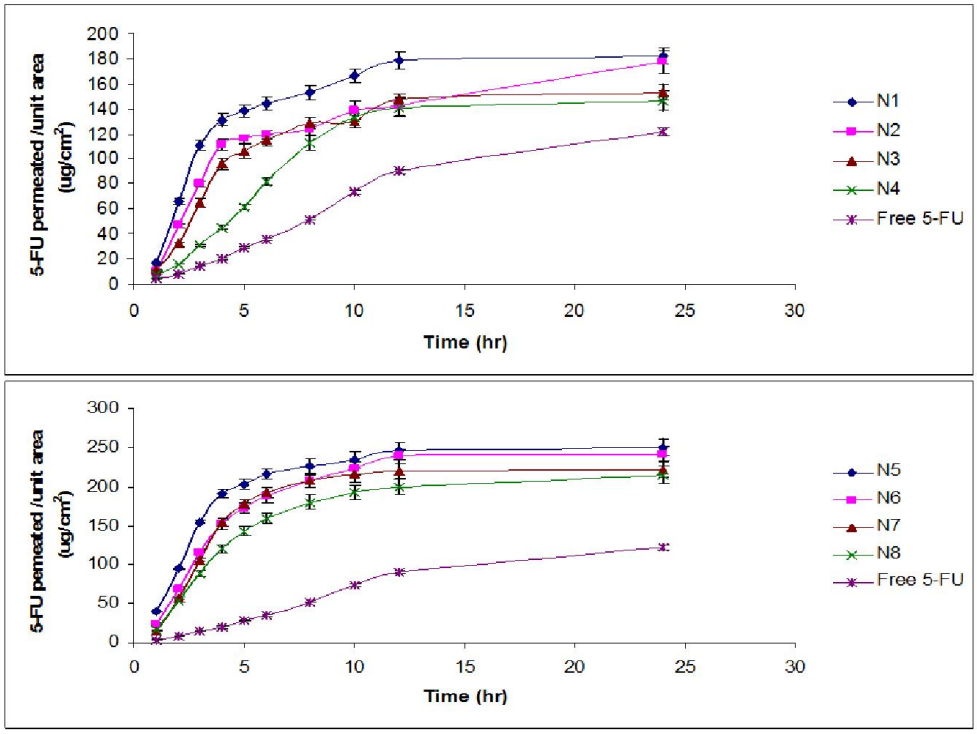

Figure 2: Permeation profiles of 5-FU from 5-FU loaded niosomes along with free 5-FU.

The in vitro diffusion studies of 5-FU gel formulae showed that 5-FU loaded niosomes' gels had higher Q_{24} of 5-FU than other formulae containing plain 5-FU due to their lipophilic nature so it can pass through lipophilic membranes adequately, this is in contrast to the hydrophilic 5- FU. Permeation parameters of different 5-FU gel formulae are shown in Table (4). Nevertheless; the amount of 5-FU diffused from 5-FU gel formulae were slightly smaller compared to those diffused from 5-FU niosomes, this could be explained by the hindrance to diffusion afforded by the viscous gel matrix The highest values were observed in case of formula composed of 5-FU loaded niosomes in NaCMC polymer, this can be attributed to repulsion between the negatively charged niosomes and the negative nucleus of the polymer. However it is also observed that drug diffused is some what greater in case of chitosan formula containing 5-FU than formula containing 5-FU loaded niosomes in chitosan, this might be due to repulsion between the acidic drug and the acidic chitosan gel, where as there could be some kind of attraction between the negatively charged niosomes and the acidic chitosan gel. Figure (3) illustrates diffusion profiles of 5-FU from different gel formulae.

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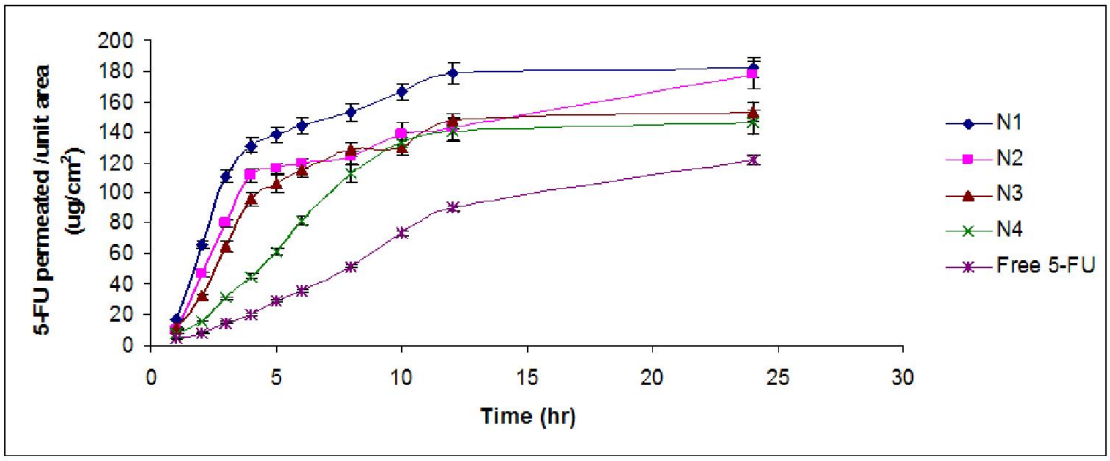
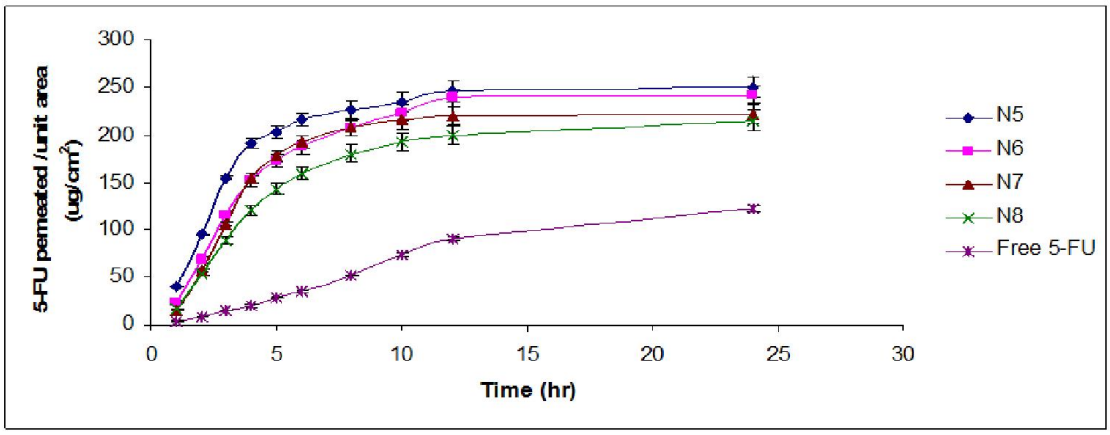


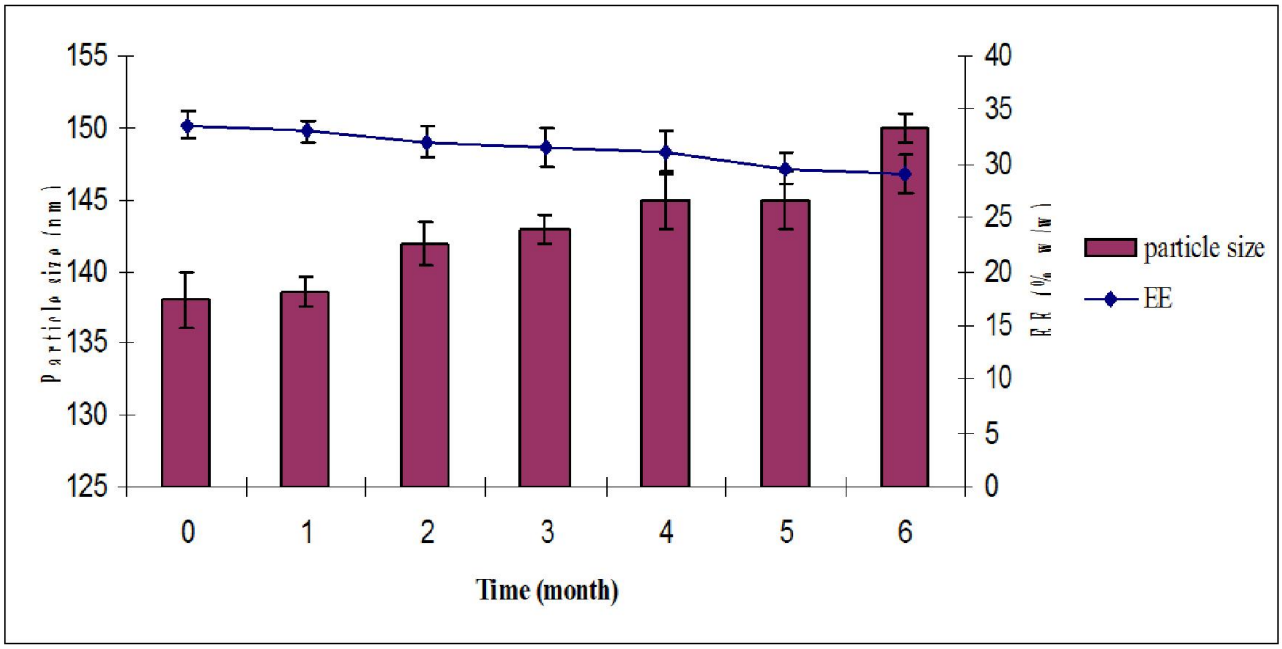


From the obtained data, the formula containing 5-FU loaded niosomes respectively in NaCMC were chosen to perform the in vivo study against free 5-FU in the same matrix.

Stability study

It was observed from figure (4) that formula N8 exhibited a decrease in entrapment efficiency (EE) (% w/w) and increase in

particle size (nm) during the first three months of storage period. These changes were found to be insignificant using One-Way ANOVA. However, after six months of storage the decrease in encapsulation efficiency and increase in particle size were found to be significant. This means that up to three month of storage the prepared niosomes formula was somewhat stable however after six months of storage the formula was unstable concerning EE and particle size.



In-vivo study

Figure (5 B) shows that the tumors induced by EAC cells at the site of injection in the negative control slide was very prominent and showed fast growth in addition to sever hemorrhage and inflammatory reaction when compared with normal skin which show normal epidermal and dermal structure with no inflammatory reaction (Figure 5 A), these results come in agreement with what is reported in literature [22]. It was found that administration of free 5-FU as a topical preparation caused a little decrease the inflammatory reaction and hemorrhage induced by EAC as the slide is still showing clear hemorrhagic patches and inflammation (Figure 5 C).

It was also found that topical application of 5-FU loaded niosomal gel (Figure 5 D) markedly decreased the inflammatory reactions and hemorrhagic patches previously noticed in negative control slide and 5-FU treated slide, which means that niosomes could deliver 5-FU more deeply compared to 5-FU gel and hence can improve the penetration of 5-FU into deeper layers of skin. These findings come in agreement with our in vitro diffusion studies which showed the efficiency of niosomes in delivering the hydrophilic therapeutic agent 5-FU to a higher extent than that of the 5-FU by itself. The significant reduction in the inflammatory reaction can be detected qualitatively under the microscope. This effect is probably related with the angiogenesis and growing factors induced by inflammation that are necessary for tumor development. The Ehrlich tumor implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an intense hemorrhagic areas formation, cellular migration and a progressive ascetic fluid formation [23]. However, the niosomes loaded with the drug were able to reduce the inflammatory reaction, hyperkeratosis and were able to reduce the angiogenesis that is a feature of proliferation of new turmeric structure.

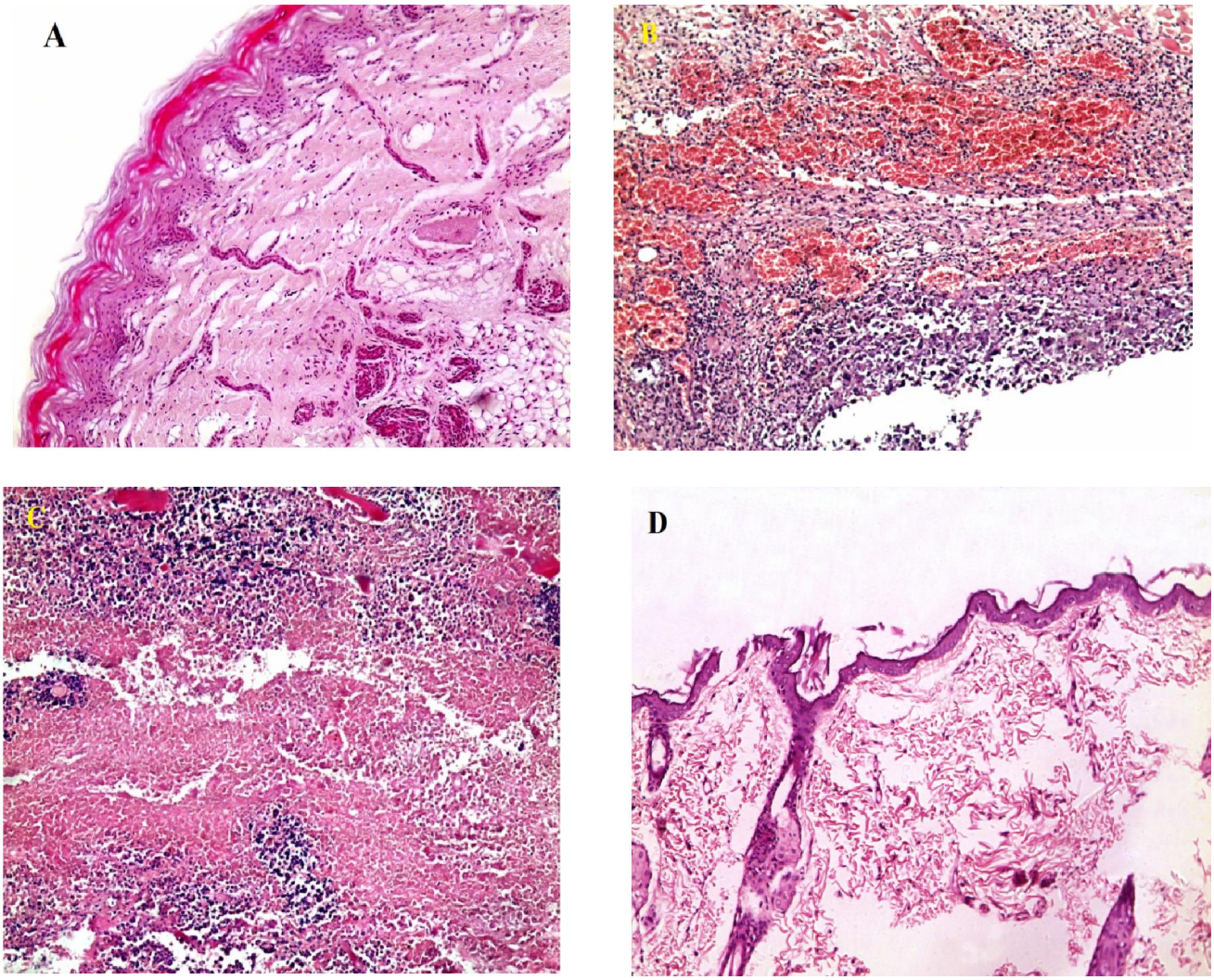


Figure 5: Cross sections through A) Normal mouse skin, B) Un treated skin tumor C) Skin tumor treated with free 5-FU gel D) Skin tumor treated with Niosomal 5-FU gel (at mag. Power 10x).

Conclusion

This study proved the ability of 5-FU to be incorporated in niosomes core with reasonable entrapment efficiency. The produced niosomes had particle size and zeta potential values that fall within the acceptable limits. All niosomes formulae had better diffusion compared to free drug. Gel matrix composed of NaCMC had the best permeation parameters and used for *in-vivo* study. The Histopathological studies proved the greater ability of niosomal gel formulae to manage skin carcinoma compared to free 5-FU.

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