

Original Article

Topical drug delivery of 5-fluorouracil proniosomal gel for the treatment of skin cancer: *in vitro* and *in vivo* evaluation

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ABSTRACT

The contemporaneous work was intended to investigate the efficacy of proniosomal gel as a topical vehicle for 5-Fluorouracil (5-FU) in the treatment of melanoma. 5-FU loaded proniosomal gel was formulated by the coacervation phase separation technique using various non-ionic surfactants (spans). The formulations were examined for different parameters such as particle size, drug percentage of entrapment efficiency (% EE), *in vitro*, *ex vivo* drug release, *in vitro* cytotoxicity and *in vivo* animal model study. The obtained % EE results for prepared proniosomal 5-FU gel in an acceptable range of desired sustaining effect. The cell toxicity in A-375 human melanoma cells revealed concentration mediated cell death. The % cell viability of A-375 human melanoma cells following loaded 5-FU gels and placebo gel treatment concentration at 10 µg/mL for 24 hr incubation was 35.6±1.9% and 15.1±2.5% respectively. The prepared proniosomes with penetration enhancer were better than the pure drug. The flux value found from 5-FUPG-11 (23.9 µg/cm²/hr) was 4 times greater than that of the drug suspension (6.6 µg/cm²/hr). There is no substantial change witnessed in vesicular size or % EE of the preparations when stored at 2-8 °C and 25±2 °C for 90 days. The outcomes of biochemical assessment also showed that the 5-FU proniosomal gel formulation can be a good carrier choice for carrying the drug into skin layers and achieving the desired sustainment effect for managing of melanoma treatment through topical delivery of a 5-FU proniosomal formulation.

1. INTRODUCTION

Skin is the outermost organ of the body, and its main functions include protection from injury, pathogens, maintains the body temperature and fluid retention. Skin is affected by many disorders, cancer is one among them. Skin cancer may be a deadly disorder with a higher incidence of cutaneous melanoma and non-melanoma carcinoma that has increased quite higher worldwide since the 1940s³.

There are two types of carcinoma which are melanoma and non-melanoma carcinoma (NMSC). Non-melanoma carcinoma is that the most often diagnosed sort of cancer, but it's the lower death rate while melanoma though infrequently diagnosed features

a high death rate⁴. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the NMSCs, and that they originate from epidermal keratinocytes⁵. Cutaneous malignant melanoma (CMM) originates from melanoma cells, which also are found within the epidermis⁴. Actinic keratosis and Bowen's disease though not true aggressive carcinomas are usually included in discussions about carcinoma due to their relationship with skin cancers⁶. Early detection of carcinoma is advocated for because neglected NMSCs may grow invasively and SCC may metastasize⁴. Prompt detection will cause early treatment and successively improved prognoses⁶. Currently, surgery is that the main recommendation for the treatment of skin cancer; however, cases do exist where surgery is not feasible and an alternate method must be used. Seeing as NMSC and CMM occur inside the skin, it falls reasonably those effective topical medicaments utilizing low adverse effect profiles are often viable replacements. Cancer is regarded as by evasion of apoptosis or defective apoptosis mechanisms which successively allow uncontrollable growth of cells⁷. Chemotherapy of cancer is thus aimed toward restoring the apoptosis balance or eliminating the speedily dividing cells⁸. Chemotherapy is one of the foremost therapeutic approaches for the management of malignancy in addition to surgery, radiation and biologic therapies (immunotherapy and hormonal therapy)^{1,2}.

5-FU is a pyrimidine antimetabolite that acts by inhibiting thymidylate synthase (TS). Thymidylate synthase is important for the replication and repair of deoxyribonucleic acid (DNA), and its inhibition causes the induction of apoptosis and cell death⁹. The uncontrolled division of cancer cells is linked to lacking apoptosis, so a rise in apoptosis should replicate an anti-tumour activity. 5-FU is a hydrophilic anti-neoplastic agent with a plasma half-life of 10–20 min^{10, 11}. To focus on target tumour cells within the skin and minimize systemic and topical adverse effects; 5-FU must enter into the skin layers and exert its effects within the epidermis and dermis. The accessible routes for administration of 5-FU are the intravenous and topical routes⁹. In this study, the main target was on the topical route of administration. Formulations of topical treatment modalities are interesting due to the relatively impermeable nature and barrier properties of the skin¹². The outermost layer of

the skin (the stratum corneum) is lipophilic, so it hinders the permeation of hydrophilic drugs like 5-FU through the skin. The physicochemical properties of API play an enormous role in determining whether or not it will diffuse into and through the skin.

Accordingly, niosomes which are surfactant based vesicles that are mostly chemical stable and fewer expensive than liposomes were introduced^{13, 14}. However, even though niosomes exhibit more chemical stability during storage, there could also be a physical stability problem upon the storage of niosome dispersion. Proniosomes were prepared as a dry powder for reconstitution before use as a way of preserving the chemical and physical integrity of vesicles¹⁵. For the topical delivery drive, proniosomes were prepared as gel-like concentrated niosomes suitable for topical application¹⁶. These gel-like structures have the advantage of being proper for scaling up while keeping the skin penetration enhancing abilities and better physicochemical stability. However, the available data on proniosomes are rare and are not systematic with numerous factors requiring further study. These factors include the effect of composed ones and therefore the likelihood of outmoded skin penetration relishes into proniosomes.

The existent work was intended to research the practicality of employing proniosomal gel as dermal delivery for (5-FU). The effect of the varied surfactants and co-surfactants was assessed on the features of proniosomes. They were in terms of vesicle size and size distribution, surface charge, % EE, and morphology. The drug release report of the number of formulations was determined by *in vitro* and *ex vivo* studies. Permeation of the proniosomes through mice skin was evaluated and thus the skin irritation was also assessed to make sure the biocompatibility. The proniosomes were further exposed to stability study findings.

2. MATERIALS AND METHODS

2.1. Materials

5-Fluorouracil (5-FU) was received as a gift sample from Mylon Company, Mumbai (India). Soya Lecithin was kindly offered by sigma chemicals limited (Hyderabad, India). Cholesterol was obtained from the Finar chemical laboratory. Span 20, span 40, span 60, span 80 grades were gotten from

Molychem labs, Thane (India). Dialysis membrane (12,000-14,000 M.W cut off) was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, (India). All additional chemicals and solvents used were of analytical grade.

2.2. Animals

Swiss albino mice (25±30 gr) 6-8 weeks old were gotten from the disease-free animal house National Institute of Nutrition, Hyderabad (India). Animals were housed in groups of 4 in cages at an ambient temperature of (20 – 25 °C) and had free access to diet and water. The research study and testing activities were reviewed and approved by the Institutional Animal Ethical Committee (Approval no.: IAEC/GIP-1287/GVR-F/2019).

2.3. Preparation of 5-FU Proniosomal Gel (5-FUPG)

The proniosomal gel was prepared using

coacervation phase separation method¹⁷ with various grades of non-ionic surfactants, lecithin, and cholesterol¹⁸. Accurately weighed the batch quantity of 5-FU along with surfactant; lecithin and cholesterol were mixed with 2.5 ml of ethanol in a round bottom flask. The contents are subjected to heat in a water bath at a temperature of 65 ± 5 °C for 5 min. To the above mixture, 1% isopropyl myristate (IPM) and 1.6 mL of pH-7.4 phosphate buffer solution were added under constant heating conditions until the formation of transparent dispersion. The transparent dispersion was subjected to cooling to room temperature until the formation of a gel like structure (proniosomal gel). Different grades of spans were utilized in the preparation to study their effect on formulation characteristics. For further optimization, different proniosomal formulations were prepared by varying the quantity of surfactant and lecithin and 1% IPM (permeation enhancer) concentration was constant in all formulations. The compositions of formulations are listed in Table 1.

Table 1. Composition of 1% 5-Fu Proniosomal Gel (5-FUPG)

Formulation	5-FU (mg)	Span 20 (mg)	Span 40 (mg)	Span 60 (mg)	Span 80 (mg)	Lecithin (mg)	cholesterol (mg)	IPM (mg)
5-FUPG-1	10	232	-	-	-	696	52	10
5-FUPG-2	10	-	232	-	-	696	52	10
5-FUPG-3	10	-	-	232	-	696	52	10
5-FUPG-4	10	-	-	-	232	696	52	10
5-FUPG-5	10	464	-	-	-	464	52	10
5-FUPG-6	10	-	464	-	-	464	52	10
5-FUPG-7	10	-	-	464	-	464	52	10
5-FUPG-8	10	-	-	-	464	464	52	10
5-FUPG-9	10	696	-	-	-	232	52	10
5-FUPG-10	10	-	696	-	-	232	52	10
5-FUPG-11	10	-	-	696	-	232	52	10
5-FUPG-12	10	-	-	-	696	232	52	10

2.4. Characterization of 5-FU Proniosomal Gel (5-FUPG)

2.4.2. Particle Size Distribution and Zeta Potential

Malvern-zeta sizer (Nano ZS90) equipment is used to determine the mean vesicle size, size distribution and followed of zeta potential through

dynamic light scattering technique. The prepared proniosome gel up to 0.2 g was dispersed in 10 ml of 7.4 pH phosphate buffer solution. The prepared solution was evaluated for size and polydispersibility index at 25°C with a refractive index of 1.333. PI is obtained as:

$$PI = (SD / \text{Vesicle size})$$

Small values of PI (<0.1) indicate a standardized population, while PI values >0.3 points too high heterogeneity.

2.4.2. Encapsulation efficiency (% EE)

To determine the entrapment efficiency of prepared proniosomal preparation Proniosomal gel (0.2g) was dispersed with 10ml of pH 7.4 phosphate buffer. The aqueous dispersion of niosomes was subjected to sonication for 30 min in a bath sonicator. The dispersion is subjected for centrifugation at 9000 rpm at 4 °C for 45 min to separate untrapped 5-FU. The supernatant was collected and the 5-FU concentration in the resulting solution was analyzed by a validated analytical method. The percentage of drug encapsulation was calculated from the below equation:

$$\% EE = (C_t - C_f) / C_t \times 100$$

Where, C_t is the concentration of 5-FU and C_f is the concentration of free 5-FU

2.4.3. Optical Microscopy

The 5-FU proniosomal gel preparations were evaluated under optical microscopy to verify the formation of niosomes upon hydration. A hydrated proniosomal gel was examined under a compound microscope at 100 X magnification.

2.4.4. Morphology characterization using scanning electron microscopy (SEM) and transmission electron microscope (TEM)

The morphology evaluation of the prepared niosomal gel was performed assessed using SEM. Sample preparation was done by hydrating proniosome gel (0.2 g) with 10 ml phosphate buffer solution (pH 7.4). The droplet of dispersion was placed on an adhesive carbon tape mounted on an aluminum stub. The samples were coated with gold employing a vacuum evaporator and examined at 25 kV accelerating voltage under SEM equipped with a digital camera¹⁹. For TEM analysis a droplet of dispersion was placed onto a carbon-coated copper grid and left to stick on the carbon substrate for around 1 min. The dispersion in additional was removed by a bit of Whatman paper. A droplet of 1% phosphor tungstic acid

solution was added and, again, the solution in excess was detached by a tip of Whatman paper. After being marked, samples were allowed to dry at room temperature for 10 min for examination²⁰.

2.4.5. Differential scanning calorimetry (DSC)

To examine the possible interactions between 5-FU and ingredients utilized in formulation development, thermal properties of pure drug and the preparations were evaluated by differential scanning calorimetry (DSC) employing a diamond (DSC) (Mettler star sw8.10)²¹. The analysis was performed at 50°C to 400°C temperature range under a nitrogen flow of 50 ml/min selected formulation with highest % EE was chosen and samples of 4 mg of each of 5-FUAPI, S60, placebo, IPM, cholesterol, lecithin and drug-loaded proniosomes-derived niosomes (5FUPG-11) were used for DSC analysis. The exact weighed amount of sample was crimped in DSC aluminum pan and samples analyzed up to a temperature of 400°C at a heating rate of 10°C/min.

2.4.6. Fourier Transforms Infrared (FTIR) Spectroscopy

The 5-FU powder was compacted into a pellet along with KBr using a hydraulic press²². The Infrared spectrum of API of 5-FU and final selected formulation was analyzed in the wave-number region of 4000-400 cm^{-1} on Fourier transform infrared spectroscopy (FTIR).

2.4.7. In vitro drug release study

The release of 5-FU from Proniosomal gel was determined by employing a dialysis membrane diffusion technique²³. An open glass tube having a diameter of 4.153 cm^2 with an efficient length of 8 cm was closed one end with the dialysis membrane previously soaked in pH phosphate buffer 7.4 overnight which acts as a rate-limiting membrane. The glass tube was filled with 5 mg gel formulation towards the donor compartment. The glass tube was placed during a beaker containing 40 ml of pH 7.4 phosphate buffer which acts as a receptor compartment medium, the entire assembly was fixed in such a way that the lower end of the tube containing membrane which upon having gel was just touched (1-2 mm deep) the surface of the

diffusion medium. The temperature of the receptor department maintained at $32 \pm 2^\circ\text{C}$ and therefore the medium was agitated at 100 rpm speed using a magnetic stirrer for 24hr. Samples were collected at different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 12, 18 and 24 hr). Aliquots of 5 ml samples were withdrawn periodically and replaced with an equivalent volume to maintain sink conditions. The collected samples were analysed using a validated analytical method ($n=3$).

2.4.8. *Ex vivo skin permeation and drug retention studies*

The animal experimentation was administered after approval by the institutional animal ethical committee (IAEC/GIP-1287/GVR-F/2019). Swiss albino mice (25-30 gr) were utilized in the study, the animals were housed in separate cages and well-maintained under the controlled conditions of temperature and therefore the mice had free access to water and food until they were sacrificed for skin harvesting Euthanasia and of the carcass as per the rules. The mice were sacrificed by using an excess amount of anaesthetic ether. Before surgical removal of the skin, the hair on the dorsal side was removed with hair clipper taking extreme precautions to not damage the skin^{24, 25}. The subcutaneous fat was removed and skin was rinsed with phosphate buffer saline, then after wrapped in aluminium foil and stored at -20°C till further use²⁶ (used within 2 weeks of the period). Ex vivo permeation studies were carried out using unjacketed vertical Franz diffusion cells with a diffusional surface area of 4.153 cm^2 and 20 ml of receptor cell volume. The skin was brought to room temperature and mounted between the donor and receiver compartment of the Franz diffusion cell, where the stratum corneum side was faced towards the donor compartment. Before being dosed the skin was permitted to equilibrate for 1 hr and formulation equal to 10 mg of 5-FU was placed on the dorsal side of the skin. The receptor compartment containing pH 7.4 phosphate buffers (containing 0.02% w/v of ethanol to retard microbial growth²⁷) was maintained at $32 \pm 2^\circ\text{C}$ under constant stirring up to 24hr. The receptor compartment was continuously stirred at 300 rpm²⁸. The donor compartment and the sampling port were covered with a lid to prevent

evaporation throughout the study. Aliquots of 5 mL were withdrawn periodically at different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 12, 18 and 24 hr) and replaced with an equal volume to maintain sink condition. The same procedure was done with pure drug and the samples were suitably diluted and the amount of drug was resolved, analysed by validated analytical method ($n=3$).

Determination of the drug retained within the skin was done by following the tactic with a small modification reported method²⁹. The experiment was carried out after completion of permeation studies 24 hr³⁰. The gel present on the compartment cell was removed and washed with PBS and methanol (1:1 v/v) and determined by the validated analytical method. The leftover skin was cut into small pieces to determine the quantity of drug in viable skin (epidermis and dermis). They were transferred into a conical flask comprising 50 mL of PBS (7.4 pH) and methanol (1:1 v/v), drug was extracted by subjecting to bath sonication. The solution was separated from the skin and the amount of 5-FU (retained in skin) content was analyzed by a validated analytical method.

2.4.9. *Skin permeation data analysis*

The cumulative amount of drug penetrated through a unit area of skin was plotted as a function of time.

Flux

The Steady-state Flux was estimated by using the slope of the graph

$$J_{ss} = \left(\frac{dQ}{dt} \right) \times \left(\frac{1}{A} \right)$$

Where J =Flux ($\mu\text{g}/\text{cm}^2/\text{h}$), A =Surface area, dQ/dt = Cumulative amount permeated per unit area per unit time.

Permeability Coefficient (KP)

Permeability coefficient which signifies the correlation between the flux and initial drug load was calculated using the following equation³¹.

$$KP = J_{ss}/C$$

Where, KP =Permeability coefficient (cm/h), J = transdermal flux, C = Primary concentration of drug in the donor compartment.

2.4.10. *In vitro* cytotoxicity study of 5-FU Proniosomal gel (5-FUPG)

The *in vitro* cytotoxicity for the free drug and drug-loaded gels were estimated by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay. Momentarily, A-375 human melanoma cells were seeded into 96-well plates in 100 μ L DMEM medium at a density of 1×10^4 cells/ well, and incubated overnight. Then, cells were exposed to a sequence of free drug and drug-loaded gels at changed strengths of 0-100 μ g/mL for 24 hr, correspondingly. A-375 human

melanoma cells without any treatment were used as the control. After the incubation period, the media were removed and therefore the cells were treated with an MTT solution (50 μ L; 5 mg/mL) in serum-free DMEM for 4 hours. The solution was removed and 150 μ L of dimethyl sulfoxide (DMSO) was added onto to each well. Formerly the plate was incubated for 30 min at room temperature. Absorbance intensity was measured by using a UV-Vis spectrophotometer at 590 nm with a reference wavelength of 620 nm. Cell viability was expressed by the subsequent equation.

$$\text{Cell viability (\%)} = \text{Absorbance of } \frac{\text{sample}}{\text{Absorbance}} \text{ of control} \times 100$$

2.4.11. *In vivo* efficacy study

Momentarily, depilatory cream was applied to require away the hair from the rear of every mouse and was left untreated for two days. Mice having no hair growth after 2 days were then selected for the experimental study³². The animals were separated into three groups of six in each. Group-I (normal control group) was kept in normal condition with none treatment. Group-II (negative control group) received DMBA and TPA (7, 12-dimethylbenz (α) anthracene), (12-O-tetradecanoyl-phorbol-13-acetate) topically for 9 weeks without treatment to induce tumour growth³³. The induced areas were visually examined for indications similar to skin cancer. Group-III (test group 1) received (1%) selected proniosomal topical gels after 6 weeks of inducing with DMBA and TPA. For every four days, the tumour volume was examined and used as a parameter for *in vivo* anti-tumour activity. It will be continued until the day of the 30th during the administration period. Body weight of animals was supervised during the treatment period. After the treatment period animals were sacrificed with high dosages of chemical with ether. The capability of the selected formulation to heal the tumour was calculated subsequently.

2.4.12. Histopathological study

After sacrificing the animal, the skin from the skin tumour site was taken. Skin biopsies were fixed at 10% buffered formalin, fixed in paraffin and sectioned at 6–10 mm. Segments were then stained with hematoxylin and eosin stain (H&E

stain) and imagined by a light microscope (Olympus, Delhi) at 80X magnification³⁴.

2.4.13. Stability Studies

The preparations were filled in to glass vials, subjected to stability studies at accelerated storage conditions ($40^\circ\text{C} \pm 2^\circ\text{C}$), room temperature ($25 \pm 2^\circ\text{C}$), and in the refrigerator ($2-8^\circ\text{C}$) for three months. The samples were analysed for entrapment efficiency and size at definite time intermissions (1, 2 and 3 months). Formulations that retain its entrapment competence for 3 months duration considered as long-lasting formulation. At the same time below samples were withdrawn and hydrated with phosphate-buffered saline solution (pH 7.4) and observed for any trace of drug crystallization under an optical microscope³⁵.

3. RESULTS AND DISCUSSION

3.1. Preparation of 5-FU Proniosomal Gel (5-FUPG)

In the present investigation 5-FU loaded niosomal gel were prepared using non-ionic surfactants using coacervation technique. The prepared niosomes were evaluated for size, shape, entrapment, *in vitro* drug release, *ex vivo* drug release and its efficacy in DMBA and TPA induced melanoma.

3.2. Characterization of 5-FU Proniosomal Gel (5-FUPG)

3.2.1. Particle size distribution and zeta potential

The particle size results freshly reconstituted dispersions of 5-FU proniosomal developed formulation are depicted in Table 2. All formulations were found in nanosize range between 125.47 nm (5-FUPG-3) and 920.34 nm (5-FUPG-10) with a low polydispersity index, ranging from 0.146 (5-FUPG-5) to 0.255 (5-FUPG-9), indicating a homogenous dispersion. Additionally, the measured zeta potential values of the formulated proniosomes

were negatively charged, with zeta potential charges ranging from -24.35 mV to -62.34 mV showing good physical stability of niosomal dispersion. Spans are the favoured non-ionic surfactants used as vesicles forming agents within the preparation of niosomes and proniosomes. On evaluating different grades of spans e.g. 20, 40, 60 or 80, it had been found that their nature considerably affected the ultimate vesicle sizes.

Table 2. Particle size, poly dispersepsity index and zeta potential analyses of 5-FU proniosomal gels.

Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)
5-FUPG-1	193.45	0.196	-24.35
5-FUPG-2	203.65	0.178	-26.48
5-FUPG-3	125.47	0.179	-28.69
5-FUPG-4	264.37	0.165	-32.95
5-FUPG-5	885.25	0.146	-55.52
5-FUPG-6	320.56	0.253	-41.31
5-FUPG-7	226.86	0.245	-56.42
5-FUPG-8	368.59	0.212	-49.82
5-FUPG-9	444.38	0.255	-37.99
5-FUPG-10	920.34	0.201	-42.34
5-FUPG-11	465.80	0.215	-62.34
5-FUPG-12	540.10	0.175	-35.80

3.2.2. Encapsulation efficiency (EE)

The encapsulation efficiency is an important parameter in designing vesicular preparations. Vesicle encapsulation ability depends on the character and amount of use of surfactant cholesterol and lecithin. The encapsulation efficiency of various preparations showed in Table 3. Higher encapsulation efficiency was showed for formulations prepared with span 60. Span 60 has the lengthiest saturated alkyl chain, exhibits higher phase transition temperature (T_c) that reveals the percent of encapsulation efficiency. Altogether different spans have the same head group but, vary in alkyl chain. Increasing the alkyl chain length leads to greater encapsulation efficiency. The encapsulation efficiency followed the manner of S 60 (C18) > S 40 (C16) > S 20 (C12) > S 80 (C18). Span 60 and 80 have similar head groups, but span 80 has an unsaturated alkyl chain. Also, span 80 has the least transition temperature ($T_c = -12^\circ\text{C}$) between all tested spans (16°C for S 20, 42°C for S 40, and

53°C for S 60). The span having the maximum phase transition temperature offers the highest entrapment for the drug and least for low phase transition temperature⁴³. Initially, at lower, middle and higher surfactant concentrations of Span 20, the % EE was found to be 81.1% (5-FUPG-1), 81.9% (5-FUPG-5) and 81.2% (5-FUPG-9). Similar to this for Span 40 at lower, middle and higher concentrations the % EE was found to be 86.3% (5-FUPG-2), 88.5% (5-FUPG-6) and 87.8% (5-FUPG-10). Span 60 at lower, middle and higher concentrations the % EE was found to be 90.4% (5-FUPG-3), 90.6% (5-FUPG-7) and 94.2% (5-FUPG-11). Among all Span 80 showed lower % EE of 80.5% (5-FUPG-4), 80.2% (5-FUPG-8) and 80.3% (5-FUPG-12) at lower, middle and higher concentrations. Span 60 showed higher % EE compared to Span 20, 40 & 60. A considerable boom in % EE becomes found for Span 60 at lower, middle and higher levels of lecithin content which is normally delivered to increase the balance of

the components. Among formulations, 5-FUPG-11 showed a higher % EE of 94.2% this formulation was selected for further characterization.

3.2.2. Optical microscopy

The formed proniosomal vesicles were

observed under a light microscope with 100X magnification to confirm their formation. The niosomes were originated to be unilamellar spherical vesicles with a smooth surface and were discrete and separate with no aggregation or agglomeration shown in Figure 1.

Table 3. Entrapment efficiency, *in vitro* and *ex vivo* percentage cumulative drug release of 5-FU proniosomal gels.

Formulation	Entrapment Efficiency (%)	<i>In Vitro</i> Percentage Cumulative Drug Release (24 h) (%)	<i>Ex Vivo</i> Percentage Cumulative Drug Release (24 h) (%)
5-FUPG-1	81.1	65.1	56.9
5-FUPG-2	86.3	66.2	61.8
5-FUPG-3	90.4	71.8	62.2
5-FUPG-4	80.5	62.9	54.9
5-FUPG-5	81.9	67.8	57.4
5-FUPG-6	88.5	68.2	63.4
5-FUPG-7	90.6	73.5	64.6
5-FUPG-8	80.2	63.2	57.2
5-FUPG-9	81.2	68.9	59.9
5-FUPG-10	87.8	69.4	64.8
5-FUPG-11	94.2	75.8	68.3
5-FUPG-12	80.3	64.8	59.7

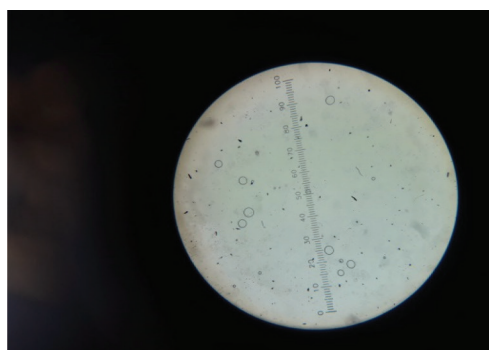


Figure 1. Optical microscopic images of 5-FUPG Formulation

3.2.3. Morphology characterization using scanning electron microscopy (SEM) and transmission electron microscope (TEM)

The surface morphology of the proniosomes was evaluated by SEM. The micrographs revealed that the vesicles were almost spherical and identifiable

with sharp boundaries and large internal aqueous space. (Figure 2. (a) & (b)). The shape and surface morphology of vesicles after hydration with distilled water for 5-FUPG-11 was shown in Figure 2(c) evaluated by TEM. The examined vesicles appeared well-identified, spherical in shape, having a smooth surface.

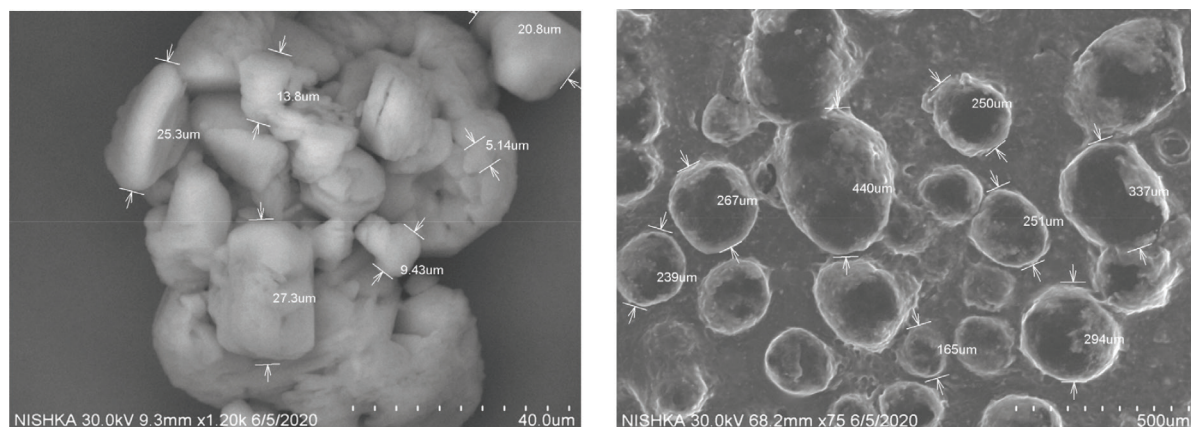


Figure 2. SEM Images of (a) 5-FU API (b) 5-FUPG-11

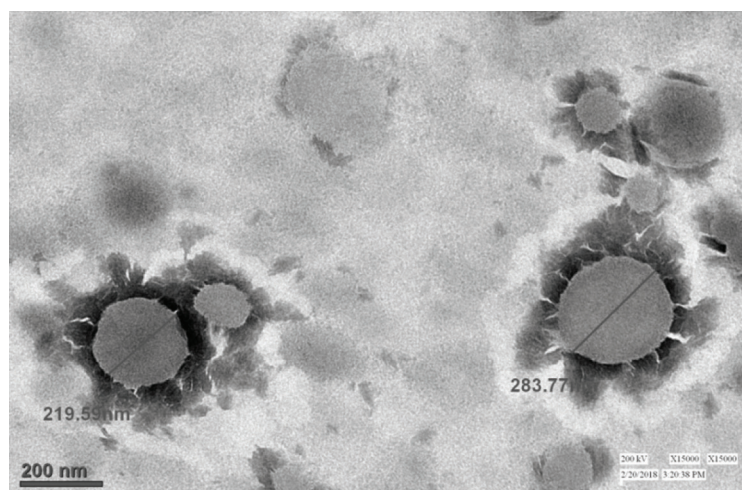


Figure 2. (C) TEM images of 5-FUPG-11

3.2.4. Differential scanning calorimetry (DSC)

DSC thermograms overlapped image of 5-FU API, other excipients and drug stacked niosomes 5-FUPG-11 are shown in Figure 3. 5-FU-delineated endothermic top at 359 °C, separately, relating to their melting temperatures. DSC thermogram of the formulation 5-FUPG-11 demonstrated a sharp endothermic top at 129 °C

showing the interaction between the molecules of the spans 60, cholesterol, and lecithin. This affirms the development of the two fold layer structure of the vesicle. No peak of 5-FU was seen in the drug-loaded formulation, demonstrating the amorphous nature of the drug. These observed results could be attributed to the complete solubility of the drug in the vesicles and can account for the enhanced entrapment of 5-FU into the formulations.

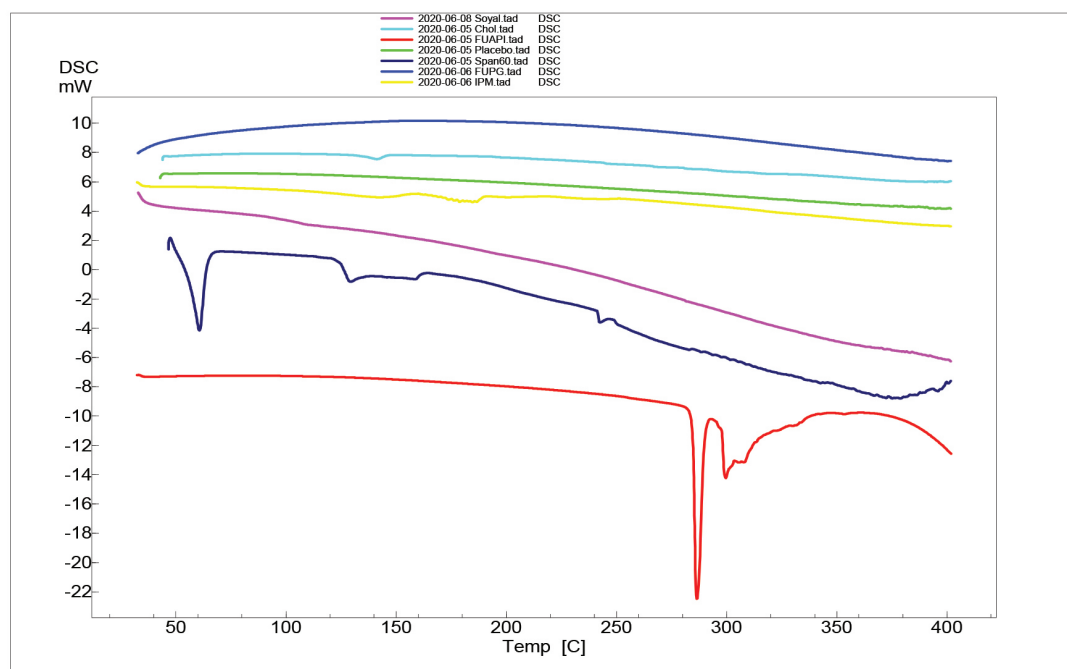


Figure 3. DSC Thermograms of 5-FU API, Soya lecithin, Cholesterol, Placebo, Span60, IPM, 5-FUPG, 5-FUPG

3.2.4. Fourier Transforms Infrared (FTIR) spectroscopy

FTIR looks at the chosen formulation (5-FUPG-11) prepared with non-ionic surfactant i.e. Span 60, soya lecithin, cholesterol together with the drug. The spectrum frequency of the formulation changed into comparable with that of the natural 5-FU, in reality, indicating that there's no drug and

excipients interaction. 5-FU showed monosubstituted alkene ($C=C$) at 756.0 frequencies whereas formulation at 761.9 observed with the aid of secondary amide ($-CONH-$) at 1680.0, in the formulation at 1707.0, cyclic alkene stretching ($C=C$) in API at 1558.54, components additionally in addition to a frequency of 1558.54. The FTIR spectra overlapped image depicted in Figure 4.

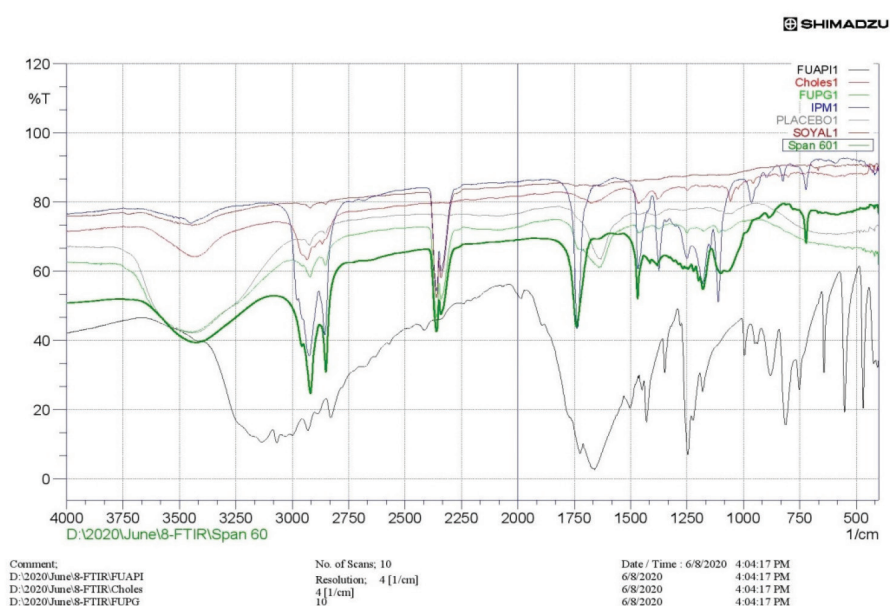


Figure 4. FTIR Spectrum of 5-FU API, Cholesterol, 5-FUPG, IPM, Placebo, Soya lecithin, Span60

3.2.5. *In vitro* drug release study

In vitro drug release indicates how the drug will perform *in vivo*. Niosomes are well-identified for being well-organized vesicles for the sustainment of drug release with a unique biphasic pattern. Values of the *in vitro* release study of 5-FU loaded proniosomal gel formulations are shown in Table 3 and Figure 5. The fusion of 5-FU in all proniosomal formulations showed a meaningfully slower release profile compared to the control of pure 5-FU release in 12hr. Early

burst effect in the first 2 hr, due to the diffusion of the surface attached drug, then followed by sustainment in the drug release, due to the diffusion of the entangled drug from inside the vesicles to the surrounding aqueous medium. Proniosomes prepared by using a higher concentration of Span 60 showed a higher release rate than those prepared using non-ionic surfactants span 20, 40 & 80. Spans 20 and 80 possess lower phase transition temperatures and formless permeable rigid bilayers than Span 60 and Span40, which forms a more permeable fluid bilayer.

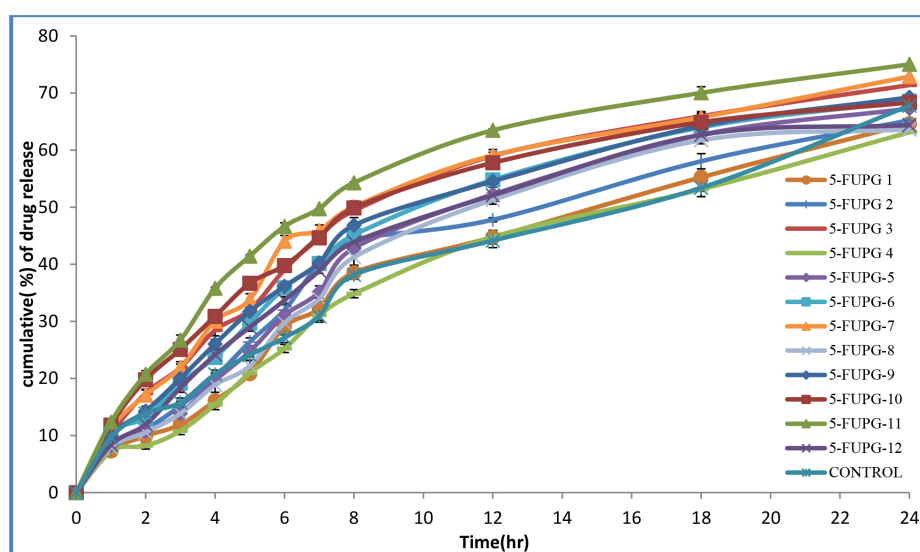


Figure 5. *In vitro* drug release of proniosomal gels (5-FUPG-1 to 5-FUPG-12) and pure 5-FU

Increasing the lecithin content from 232 to 696 mg resulted in a more intact lipid bilayer as a barrier for drug release which decreased its leakage and permeability, delay the release of entrapped drug from the vesicles and led to a significant slow release profile. Also, the best release profile among different formulations was observed upon using span 60 with a high level of 696 mg concentration. Formulation 5-FUPG-11, containing span 60 as the surfactant, is showing more *in vitro* drug release i.e., 75.8% among all the surfactants. Therefore, based on *in vitro* characterizations, the selected formula 5-FUPG-11 based span 60 was further progressed to stability Table 3.

When we compare the release patterns of the control formulation with that of proniosomal formulation, a meaningful retardedness in drug release was witnessed in proniosomes. To treat the targeted site drug delivery specifically the

treatment of skin cancer type of topical diseases, long term treatment is required with an extended-release pattern. Selected batch was directed using various models like Zero order, First order, Higuchi, Korsmeyer-Peppas. The kinetic analysis of all preparations profile followed diffusion controlled mechanism with an initial fast release phase followed by slower releases one. Release of 5-FUPG-11 batch corresponded to the Higuchi matrix equation showing a high R square value. (0.96429). The other models R^2 values of Zero order, First order, Peppas. 0.8031, 0.9344, 0.810 found respectively. Therefore, it can be fulfilled that the release of 5-FU from proniosomal gel formulation was based on a higuchian matrix diffusion-controlled mechanism. That's why; the release of drug from this matrix possibly was influenced by the diffusion of the matrix.

3.2.6. *Ex vivo* skin permeation and drug retention study

Proniosomes ought to be hydrated to shape niosomal vesicles before the drug release and penetrates over the skin. Several mechanisms could clarify the capacity of niosomes to tweak drug move crosswise over skin including (i) adsorption and combination of niosomes onto the outside of skin would make simpler drug permeation, (ii) the vesicles go about as infiltration enhancers to diminish the obstruction properties of the stratum corneum and (iii) the lipid bilayers of niosomes go about as a rate-restricting film boundary for drugs. Curiously, no slack stage could be recognized and inside one hour 5-FU could be identified in the receptor medium demonstrating that all procedures (water pervasion from the receptor compartment to the skin, proniosomes transformation to niosomes, 5-FU discharge from the reconstituted niosomes and its penetration) occurred quickly.

The significant permeation of 5-FU results just when the drug is released from the niosomes shaped after the hydration of proniosomal gel with the skin fluids. The infiltration information got from various surfactant-based proniosomal plans was contrasted and control of identical drug concentration. The outcomes uncovered that the measure of drug saturated through the skin in the main time frame was demonstrated higher. Subsequently, with every vesicular definition, the infiltration rate was moderate. Results appeared in Table 3 above and charts of Figures 6. Seems to demonstrate that proniosomal formulations unmistakably deferred the drug infiltration through the skin. A huge improvement of drug maintenance in the skin was seen with proniosomes as they comprised of cholesterol, lecithin, and a surfactant. The formulation 5-FUPG-11 (more significant level of ranges 60) indicated drug release arrival of 68.3% than other preparations with span 20, span 40, span 80.

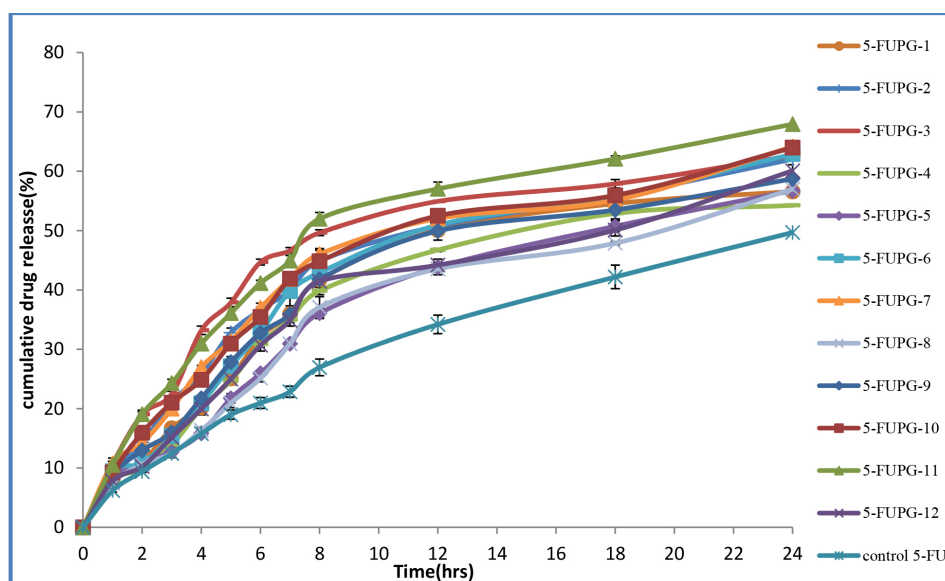


Figure 6. Ex vivo drug release of proniosomal gels (5-FUPG-1 to 5-FUPG-12) and pure 5-FU

3.2.6. *Skin permeation data analysis*

The determined permeation parameters are exhibited in Table 4. All proniosomal formulations delivered the drug through the skin at a fundamentally higher rate compared to the saturated aqueous control. This is clear from the recorded transdermal flux values which were notably higher in the case of proniosomes. Merging of lecithin

into the proniosomes resulted in a substantial increase in transdermal drug flux equated to the drug solution. The skin permeation factors of proniosomes as well as drug suspension at the equivalent drug concentration were also governed. The flux value acquired from proniosomal gel for 5-FUPG-11 ($23.9 \mu\text{g}/\text{cm}^2/\text{hr}$) was found to be 4 times greater than that of the drug suspension

(6.6 $\mu\text{g}/\text{cm}^2/\text{h}$) (Table 4). The similar low skin penetrability of 5-FU in suspension was because of its lower fluid solvency, while the higher penetrability of proniosomes brought about improved skin pene-

tration of 5-FU. Regarding the impact of infiltration enhancers, the fuse of IPM into proniosomes brought about a noteworthy increase in the transdermal flux of 5-FU in contrast with the pure drug solution.

Table 4. Permeation parameters of drug dispersion and 5-FU proniosomal gel formulation.

S. NO	Formulation	Flux($\mu\text{g}/\text{cm}^2/\text{h}$)
1.	5-FUPG-9	12.3
2.	5-FUPG-10	16.1
3.	5-FUPG-11	23.9
4.	5-FUPG-12	10.5
5	Drug dispersion	6.6

3.2.7. *In vitro* cytotoxicity study of 5-FU Proniosomal gel (5-FUPG)

The *in vitro* cytotoxicity of drug-loaded gels was assessed in A-375 Human melanoma cells. The gels without active drug with the identical polymer concentrations as drug-loaded gels made use of as control. The cells were incubated for 24 hr in the presence of the free-drug and drug-loaded gels and their survival were analysed by the MTT assay. The viability of A-375 Human melanoma cells after incubation with numerous formulations was presented in Figure 7. 5-FU loaded gels incubated for 24 hr exhibited higher cytotoxicity in A-375 Human melanoma cells compared to free

5-FU. Concentrations dependent increase in 5-FU toxicity was observed in A-375 Human melanoma cells. The % cell viability of A-375 Human melanoma cells following 5-FU loaded gels and free 5-FU treatment at 5-FU concentration 10 $\mu\text{g}/\text{mL}$ for 24 hr incubation was $35.6 \pm 1.9\%$, $15.1 \pm 2.5\%$ (Figure 7), respectively. The formulated gel was compared with the marketed gel of 5-FU as shown in Figure 7. To demonstrate that the cytotoxic effect of 5-FU proniosomal gel is because of the toxicity of 5-FU, we tried the drug-free gels for their effect on cell viability. Blank gels showed insignificant cytotoxicity in uppermost concentration.

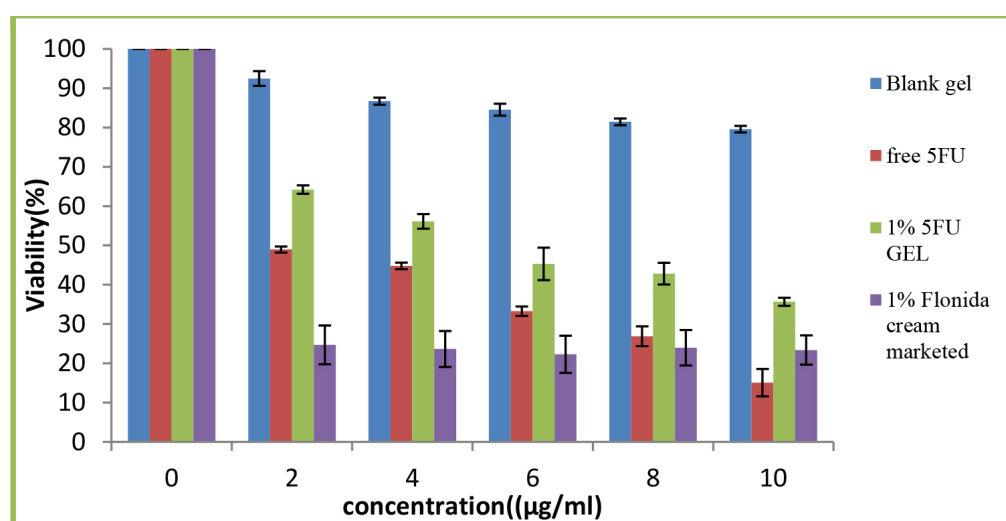


Figure 7. In-vitro cell viability data of free 5-FU and 5-FU loaded gel in A-375 Human melanoma cells culture.

3.2.8. Evaluation of *in vivo* efficacy

Results of visual perception in Figure 8. Shown that constant decrease in tumour size after the third and sixth weeks of use of the formulation III (got (1%) 5-FUPG-11 stacked proniosomal topical gel) was watched, demonstrates that the mice were treated with 1% proniosomal gels were seen with the most extreme decrease in the tumour size following a month and a half.

3.2.9. Histopathological study

Histopathological assessment of mice skin from control Group-I (normal control group) was kept in the ordinary condition with no treatment. Group-II (negative control group) got DMBA and TPA (12-O-tetradecanoylphorbol-13-acetate) topically for 9 weeks without treatment to incite

tumour development. Group-III (test group 1) got (1%) 5-FUPG-11-stacked proniosomal topical gels following a month and a half of acceptance with DMBA and TPA represented in Figure 9 (A). It was discovered that the skin of the control group has slight epidermis and ordinary skin layers. The cancer-causing agent control bunch shows moderate hyperkeratosis in the epidermis, leucocyte penetration in centre layers and moderate acanthosis as appearing in Figure 9. (B). Figure 9(C) shows the skin segments after the six week of the proniosomal gel application; the skin articulations insignificant hyperkeratosis and no acanthosis. Consequently, the after effects of the histopathology of the skin after different medicines demonstrate that the 5-FUPG-11 stacked proniosomal topical gel was progressively valuable in the treatment of skin tumours.

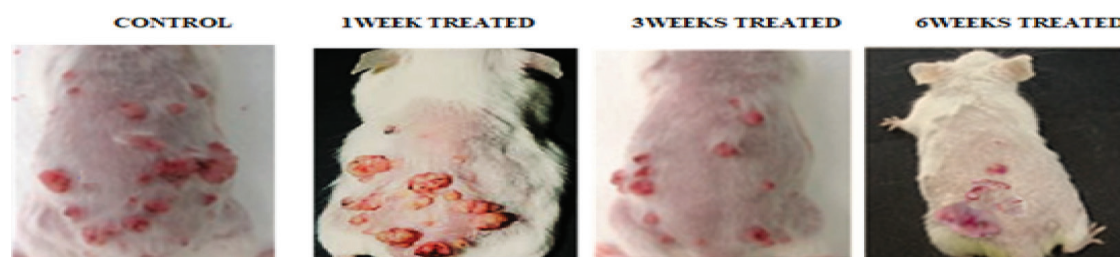


Figure 8. Tumour growth has exposed after chemical induced (DMBA and TPA) tumour to Swiss albino mice following cancer therapy procedure with normal, (A) 1-week (B) 3-weeks (C) 6-weeks treated



A

B

C

Figure 9. (A) Normal skin of mice revealing various normal layers of skin. (B) Skin of mice treated with carcinogen DMBA, TPA after 9 weeks. (C) Skin after 6 weeks' treatment with proniosomal gel, Images were observed at 100x magnification

3.2.10. Stability studies

There was no noteworthy change observed in the vesicular size or % EE of the formulations when put stored at 2-8 °C and 25±2 °C for the time of 90 days. As obvious from the outcomes, a very slight reduction in entrapment efficiency was

observed in the formulation stored in 40 °C. It was expected due to 5-FU leakage from the vesicles. The stability data are represented in Table 5. The data reveals formulation has high stability in room and refrigerator storage conditions.

Table 5. Stability study of 5-FUPG-11.

Storage time (days)	2 - 8 °C		25 ± 2 °C		40 °C ± 2 °C	
	Vesicle size (nm)	Entrapment Efficiency (%)	Vesicle size (nm)	Entrapment Efficiency (%)	Vesicle size (nm)	Entrapment Efficiency (%)
0	465.8	94.2	465.8	94.2	465.8	94.2
30	464.1	94.1	465.6	94.1	461.2	92.0
60	465.3	93.5	463.1	93.9	462.5	92.5
90	464.5	93.9	463.8	93.5	460.0	91.6

4. CONCLUSION

Proniosomes are promising transdermal delivery systems a framework with their viability is subject to their structure. The appropriate determination of the proniosome parts may prompt better use of such definitions. The surface morphology uncovered that the vesicles were practically circular and recognizable with sharp limits and huge internal aqueous space. The exploratory outcomes indicated that the % EE for the readied 5-FU proniosomal gels is adequate and they are Nano estimated and the diffusion from the gels gave the ideal sustaining impact. Formulation 5-FUPG-11, containing a higher concentration of span 60 as a surfactant, in vitro indicated more drug release i.e., 75.8% among every one of the surfactants. The formulation-5-FUPG-11 (significant level of spans 60) indicated a higher ex vivo drug release of 68.3% than different formulations with spans 20, 40, 80. Strength dependent increase in 5-FU toxicity was observed in A-375 Human melanoma cells. The % cell viability of A-375 Human melanoma cells following 5-FU loaded gels and free 5-FU treatment at 5-FU concentration 10 µg/mL for 24 hr incubation was 35.6±1.9%, 15.1±2.5%. Penetration enhancers containing proniosomes were superior to a pure drug suspension. The movement esteem got from proniosomal gel for 5-FUPG-11 (23.9 µg/cm²/hr) was seen as multiple times higher than that of the medication suspension (6.6 µg/cm²/hr). There is

no critical change seen in the vesicular size or % EE of the formulations when putting away at 2 - 8 °C and 25±2 °C for the time of 90 days. The mechanism of enhanced transdermal conveyance from proniosomes requires further examinations. The article in this manner gave a new composition to transdermal proniosomes and opened doors for future research on the enhancement of the composition together with mechanistic examinations. This examination demonstrated the appropriateness of the proniosomal gel in accomplishing the ideal sustainment impact for topical delivery of 5-FU for the administration of melanoma treatment.

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