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Original Article

Formulation, Characterization and *in vivo* Evaluation of 5-Fluorouracil-Loaded Polymeric Micelles for Non-Melanoma Skin Cancer

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ABSTRACT

Background: The goal of the study was to evaluate the prospective of fluorouracil-loaded polymeric micelles for topical delivery in non-melanoma skin cancer. **Materials and Methods:** Polymeric micelles were developed by thin film hydration technique and characterized for physico-chemical properties, *in vitro* drug release, *ex vivo* skin penetration as well as *in vivo* skin irritation and anticancer effect. **Results:** Preliminary studies were carried out in twelve Formulations (FM1-FM12) and the Formulation (FM4) with the highest entrapment efficiency (~95%) and greater drug release (~98%) was developed into gels (FG1-FG5). The selected gel Formulation (FG4) exhibited adequate viscosity (~5000 cP), ideal pH (~6.8), higher drug content (~79%), greater drug permeation (~70%) and good stability for three months. *In vivo* skin irritation results indicated the developed formulation is non-irritant. MTT assay indicates dose-dependent effect of FG4 with IC_{50} of 15.56 µg/mL. Anti-cancer activity assessed in rat models indicates the prominent reduction in tumor burden (volume) by 80% following application of fluorouracil-loaded micellar gel for 1 month. The histopathological study further confirms the activity of developed gel in melanoma skin cancer. **Conclusion:** The data observed suggests that the polymeric micelles could be a feasible option for delivering fluorouracil in the treatment of basal-cell carcinoma.

Keywords: Drug release, Fluorouracil, Polymeric micelles, Rats, Skin carcinoma.

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INTRODUCTION

The global incidence of skin cancers is rising due to prolonged exposure to sunlight, changing climatic conditions and various individual and societal factors.[1,2] Skin cancers broadly include cutaneous melanoma and non-melanoma skin cancers (keratinocyte cancers). The keratinocyte cancers comprising a group of cancers that originate in the skin's epithelial cells, primarily basal cell carcinoma (basalioma) and squamous cell carcinoma (epidermoid carcinoma). Unlike melanoma, which arises from melanocytes, keratinocyte cancers are generally less aggressive but significantly more prevalent, particularly among Caucasians, accounting for the majority of skin cancer cases worldwide.[3,4] The development of keratinocyte cancers is multifactorial, with ultraviolet radiation being the primary risk factor due to its ability to induce the malignant transformation of progenitor cells.[5] Additional risk factors include concurrent diseases such as psoriasis, chronic human papillomavirus exposure, drug-induced immunosuppression,[6] and low socioeconomic status.[7] While basalioma tends to grow slowly and rarely metastasizes, squamous cell carcinoma has a higher potential for local invasion and metastasis if untreated. Early detection and treatment, which may include surgical excision, cryotherapy, or topical therapies, typically result in excellent prognoses.[7] Public health measures emphasizing UV protection and regular skin screenings play a crucial role in reducing the burden of keratinocyte cancers.[8]

5-fluorouracil is a hydrophilic pyrimidine antimetabolite with a molecular weight of 130.08 g/mol and belongs to the BCS class

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III group indicating low membrane permeability. 5-fluorouracil acts by inhibiting thymidylate synthase, disrupting DNA synthesis and cancer cell proliferation.[9,10] Its short half-life, hydrophilicity, varying metabolic rate, severe cytotoxic effects, low oral bioavailability and quick clearance upon intravenous administration limit its clinical application.[11] On the other hand, topical fluorouracil reduces systemic adverse effects and is an FDA-approved treatment for low-risk, superficial basal cell carcinomas with numerous commercial formulations available with different drug levels (0.5-5%).[12] In topical therapy, 5-fluorouracil specifically targets rapidly growing cells in aberrant skin wherein it interrupts intracellular nucleotide reserves, which eventually causes inflammation, destruction and lesion cure. Because of its directed cytotoxicity, topical therapy is an intriguing technique in dermato-oncology, capable of treating both non-malignant as well as malignant skin problems while inflicting minimum damage to normal skin cells.[10] The commercial fluorouracil formulations are applied twice daily for 2-4 weeks, which offers a non-invasive, cost-effective alternative with minimal cosmetic impact, particularly for patients with multiple lesions. While generally well-tolerated, common side effects of topical therapy of this antitumor drug include mild erythema, local skin irritation such as burning, itching and photosensitivity.[13] Other limitations include limited skin permeability, possible systemic toxicity and variable efficacy.[14] Considering the above facts, we believe that the ideal topical therapy of fluorouracil should be a controlled and localized release formulation that could effectively deliver the therapeutic drug level in the epidermal layers.

Nanocarriers, such as liposomes,[15] transferosomes,[16] niosomes,[17] polymeric nanoparticles,[18] and nanoemulsions,[19] have been evaluated for their delivery and efficacy of fluorouracil in treating both basal and squamous cell carcinoma. The co-delivery of fluorouracil with resveratrol in a lipid nanosystem was also reported.[20] These carriers have found some promising results with improved drug diffusion through the stratum corneum, steady drug release, reduced side effects, etc.[21] In this context, polymeric micelles are nanosized colloidal carriers formed by the self-assembly of amphiphilic block copolymers in aqueous solutions.[22] Their distinct core-shell structure enables the hydrophilic molecules to be incorporated on the system's surface or in the intermediate location, while the hydrophobic core can encapsulate drugs that are poorly soluble in water.[23,24] The last two decades have seen a significant amount of research on polymeric micelles as effective and adaptable drug delivery carriers for cancer treatment.[25,26] This study hypothesized that encapsulating fluorouracil in polymeric micelles and formulating it as a topical gel offers enhanced drug penetration and local drug depot in cancerous tissues, thereby reducing systemic absorption and minimizing adverse effects. In addition, the encapsulated molecules will progressively release the drug in the target area (epidermal layers) for an extended period, which in turn reduces the frequency of application. The use of copolymer, Pluronic F127, has recently received a lot of interest in the development of dermal and transdermal delivery systems for its ability to enhance drug permeation into the skin as well its retention.[27,28] Thus, the goal of this study was to develop and assess the efficacy of polymeric micelles containing 5-fluorouracil in the treatment of non-melanoma skin cancer. Polymeric micelles containing 5-fluorouracil were developed employing the film hydration method, analyzed for pharmaceutical characteristics, drug release and transformed into a topical micellar gel with carbopol 940. The developed gel containing micelles was extensively studied for assessing the product efficacy by various methods including cell line (SH-SY5Y neuroblastoma), *in vivo* tests such as skin irritation, anti-cancer activity and histopathological evaluation.

MATERIALS AND METHODS

Chemicals

5-flourouracil was provided by Karunesh Remedies (GIDC Panoli, Ankleshwar, Gujrat, India) as a complimentary sample. Pluronic F-127 was purchased commercially from BASF Corporation (New Jersey, USA). Acetone, acetonitrile and methanol were purchased from Qualigens Fine Chemicals (Mumbai, India). Carbopol 940, sodium chloride and sodium hydroxide were purchased from Central Drug House (New Delhi, India). Sudan III dye AR, dialysis membrane (2.4 nm pore size; MW cut-off: 12,000-14,000), propylene glycol, ethylenediaminetetraacetic acid, triethanolamine, methyl paraben, dimethyl sulphoxide, foetal bovine serum (FBS), Phosphate Buffer Saline (PBS), 3-(4, 5-dimethylthiazol-2yl)-2, 5-Diphenyltetrazolium Bromide (MTT) and Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium (MEM) were purchased from Himedia (Mumbai, India).

Critical Micelle Concentration (CMC)

The CMC of Pluronic F-127 was measured using a method described earlier using Sudan III dye.[29] Briefly, the stock solution was made by adding 1% w/v Sudan III in water. Various concentrations (1-8 mg/mL) of non-ionic copolymer surfactant solution were prepared. Polymeric surfactant solution (2 mL) was mixed with 1 mL of the Sudan III Dye solution and the samples were subjected to constant shaking for 24 hr while maintain the temperature at 37°C in order to achieve equilibrium. The prepared sample's absorbance was determined at λ_{max} (520 nm) by UV/vis Spectrophotometer (Electronic India, 2375, Parwanoo, India). The CMC was determined using the X-intercept of the linear fit plot of solubilised Sudan III against polymer concentration.[30]

Preparation of Fluorouracil Loaded Polymeric Micelles

The most common method (thin film hydration) was used to develop polymeric micelles containing fluorouracil. Varying

amounts of the drug and the copolymer (Pluronic F-127) were dispersed in 20 mL of methanol, acetone, or acetonitrile. The drug concentration varied based on the formulation design or dose of the drug, as indicated in Table 1. The mixture was then sonicated for 2-3 hr at room temperature to dissolve the contents and was transferred into a 250 mL round-bottom flask. After that, the solvent was evaporated using a rotary evaporator (Hicon®, New Delhi, India) running at 120 rpm and 40-500°C. The solvent was dried within 30 min and a thin layer containing both drug and polymer was formed in the bottom of the flask.[31] The product was kept at room temperature overnight to remove residual solvents. Following that, 50 mL of distilled water was used to hydrate the thin film and the mixture was heated to 60°C and sonicated (37°C and 100 rpm) until the micellar suspension formed. The product was kept for 24 hr and the unentrapped drug was removed using a dialysis bag. The micellar suspension was filtered (0.45 µM, Sertorrius A.G. 37070, Goettingen, Germany) to get drug loaded polymeric micelles.

Evaluation of Fluorouracil-loaded Polymeric Micelles

Entrapment Efficiency

The entrapment efficiency of the drug in the formulations was assessed by adding 5 mL of ethanol (for total micelle breakdown and drug release) to 1 mL of polymeric micelles. The volume was adjusted to 10 mL with PBS (pH 6.8). The entrapment efficiency was determined spectrophotometrically at 265.2 nm and the percentage entrapment efficiency was computed using the following equation:[32]

Entrapment efficiency=(Amount of drug present in micelles/ Total drug incorporated)×100

Particle Size Characterization

The Zetasizer (Malvern Instruments Ltd., Worcestershire, UK) was used to measure the size of drug-loaded Pluronic micelles and the polydispersity index. The scattering angle was set at 173 degrees and the temperature was maintained at 25°C. A sample of polymeric micelles loaded with fluorouracil was diluted using deionized water and filtered through a membrane with a pore size of 0.45 µM and the particle size was measured. Determination of particle size was carried out with a transparent, disposable sizing cuvette.[33]

Differential Scanning Calorimetry (DSC)

The thermogram was recorded using Q20 V24.4 Build 116 DSC (TA Instruments, New Castle, DE, USA). Required amount of test products (2 mg) were weighed and directly placed in an aluminium pan and subjected to a dynamic nitrogen environment with a heating rate of 10°C per minute and scanned between 25°C and 350°C while keeping an empty pan in a similar situation as a reference.[34]

In vitro Release

The *in vitro* release of fluorouracil-loaded micelles was performed in a Franz diffusion cell using dialysis membrane. PBS (3 mL, pH 6.8) was kept in the receptor compartment and 1 mL of a formulation containing fluorouracil-loaded miceller was kept in the donor compartment. The dialysis membrane (pore size 0.22 µM) was used to divide the donor and receptor chambers.[35] A magnetic stirrer was used to continuously agitate the receiver part of the diffusion cell and the temperature was maintained at 32±1.0°C in accordance with body circumstances. At various intervals (0-10 hr), samples (1 mL) were withdrawn from the diffusion cell via the sampling port and was spectrophotometrically measured at 265.2 nm. Similar volumes of brand new PBS were added to the

Table 1: Formulation details of the fluorouracil loaded polymeric micelles carrier system.

Batch code	Fluorouracil (mg)	Polymer	CMC (mg/mL)	Organic solvent
FM1	50	Pluronic F-127	4	Methanol
FM2	100	Pluronic F-127	4	Methanol
FM3	150	Pluronic F-127	4	Methanol
FM4	200	Pluronic F-127	4	Methanol
FM5	50	Pluronic F-127	4	Acetone
FM6	100	Pluronic F-127	4	Acetone
FM7	150	Pluronic F-127	4	Acetone
FM8	200	Pluronic F-127	4	Acetone
FM9	50	Pluronic F-127	5	Acetonitrile
FM10	100	Pluronic F-127	5	Acetonitrile
FM11	150	Pluronic F-127	5	Acetonitrile
FM12	200	Pluronic F-127	5	Acetonitrile

Table 2: Composition of fluorouracil loaded gel formulation of polymeric micelles (FG1- FG5).

Code	Carbopol 940 (mg)	Methylparaben (mg)	Ethylenediamine-tetraacetic acid (mg)	Triethanolamine (mL)	Propylene glycol (mL)	Water (mL)
FG1	0.250	0.005	0.004	1.5	4	q.s.
FG2	0.300	0.005	0.004	1.5	4	q.s.
FG3	0.350	0.005	0.004	1.5	4	q.s.
FG4	0.400	0.005	0.004	1.5	4	q.s.
FG5	0.450	0.005	0.004	1.5	4	q.s.

receptor compartment following each samplingreease. The *in vitro* permeation of drug was estimated and plotted.

Transmission Electron Microscopy

The Fluorouracil-loaded polymeric Micelles (FM4) were examined morphologically using transmission electron microscopy. Briefly, on a 400 mesh copper grid coated with carbon, one drop of polymeric micelles was applied and excess samples were removed using tissue paper. A drop of a 4% w/v phoshotungstic acid solution was added to the grid and the stained sample was left to air dry.[35] The grid was examined at a 19000 X magnification by placing it in a sample holder and pictures were acquired at 200 kV of acceleration voltage.

Preparation of Micellar Gel

Carbopol 940 and methylparaben were dispersed gradually in distilled water with constant stirring for an hour. The mixture was stored at room temperature for 8 hr to allow sufficient swelling. Ethylenediaminetetraacetic acid and triethanolamine were separately dissolved, mixed for 15 min and then added to the carbopol base to neutralise it (pH 6.7-6.9). Distilled water (12 mL) was added to the propylene glycol solution, which was previously heated to 65°C in another beaker and was stirred (200 rpm) for 10 min. The propylene glycol solution was introduced drop by drop into the carbopol base while being constantly stirred. The fluorouracil-loaded miceller gel's formulation (FG1-FG5) composition was summarized in Table 2.

Characterization of Fluorouracil-loaded Polymeric Micellar Gel

Physical Properties

The physical characteristics of the prepared micellar gels with fluorouracil were evaluated visually for color, phase separation, homogeneity and clarity. The pH measurement was carried out using the Hicon® Digital pH meter (New Delhi, India). A Brookfield viscometer was utilized to determine the viscosity of gel compositions at 25°C and 12 rpm spindle speed. The plate was filled with samples (0.5-1 g) and allowed to settle until the temperature of the cone was steady. After that, the micellar gel's viscosity was monitored for 2 min.[36] The spreadability of prepared gels was measured by the protocol described earlier with minor changes.[37] Briefly, 2 g of gel formulation was applied with weight of 100 g and pressed for 5 min. The diameter of the gel spread was checked. Spreadability was measured according to the equation generally used to calculate it:[38]

$$S=m\times l/t$$

where, S=spreadability, m=weight tied to upper slides, l=length of the glass slide, t=time taken in second.

Drug Content

The prepared gel (1 g) was taken in a glass vial, added 50 mL of methanol and allowed to mix for 1 hr until the drug dissolved. The mixture was filtered with a Whatman filter paper and the samples were diluted and analyzed by UV spectroscopy.

Skin Permeation Study

Franz diffusion cell was used to determine the skin permeation of selected formulations. Porcine skin was utilised as the membrane, while PBS (pH 6.7) was utilised as the receptor medium. The skin was placed between the donor and receptor compartments in a such a way that the top hairy region has direct contact with the formulation.[39] A weighed amount of the micellar gel Formulation (FG4) or marketed formulation (Flonida 1% cream, Menarini India Pvt. Ltd., Mumbai, India) was applied to the skin. As a receptor media, 3 mL of PBS (pH 6.7) was added and the mixture was continually agitated using a magnetic stirrer. The medium's temperature was kept constant at 37±1°C. Samples were measured using UV spectrophotometer at 265.2 nm.

In vivo Skin Irritation

The skin irritation test was done on rats with the optimized Formulation of micellar gel (FG4). This test was performed to find out any skin problems after applying the micellar gel on the skin and each formulation was applied on rats for optimum results. About 1 g gel was applied topically to the dorsal side of rats for three days. Approximately 2 square inch area was covered with micellar gel and observed any lesions or irritation/redness.

Stability

The stability of the selected polymeric micelle gel (FG4) was checked for a period of 3 months by storing it in an amber-colored glass vial at 25±2°C and a relative humidity of 60%.[40] Samples

were assessed for color, phase separation, homogeneity, clarity and drug content.

Cell Line Study

The cell line used (SH-SY5Y neuroblastoma) was attained from the National Centre for Cell Sciences in Pune, India. The cells were grown in DMEM media supplemented with 10% heat-inactivated FBS and penicillin/streptomycin solution (100 units/mL and 100 mg/mL, respectively) at 37°C in a humidified, 5% CO_2 atmosphere. For cell viability assay, SH-SY5Y cancer cells (1×10^4) were cultivated in a 96-well plate and were incubated for 24 hr. Following a full day of exposure to various sample concentrations (25, 50, 100 and 200 µg/mL), the dead cells were eliminated by washing the cells with PBS. The positive control was represented by cells treated with vincristine sulphate (5 µg/mL), while the negative control was made up of untreated cells. Three duplicate runs of the MTT experiment were performed and the proportion of cell activity was determined according to an earlier study.[33]

Anti-Cancer Activity

The ethical approval for the experiment was procured from the institutional ethical committee (1355/PO/Re/S/10/CPCSEA) with protocol no. MMCP-IAEC-102. Albino rats of 8-10 weeks of age were obtained from the Animal House at Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala and kept in compliance with the guidelines for the protocol issued by the Institutional Animal Care and Use Committee. The rats were kept in a 12-hr light/dark cycle at 25°C. The dorsal skin of the rats (3×3 cm) was shaved using hair trimmer. The rats used for the anticancer activity protocol were then divided into two groups: Group A, animals received no carcinogenic products (control group, n=3) and Group B, animals received a single dosage of 7-12-Dimethyl-1-benz (a) anthracence (DMBA), then one week later, 12-O-tetradecanoy-1-phorbol-13-acetate (TPA, 62.5 µg in 500 µL) was applied twice a week (n=12) for 6 weeks. Tumor induction was done using DMBA (as initiator) and TPA (as a promoter).[41,42] Throughout the trial, the rats' body weight, tumour area and volume were checked every week for 6 weeks. After confirming the tumor development, selected Formulation of micellar Gel (FG4) was applied for Group B daily for one month.

Histopathological Study

After the sixth week of anti-cancer activity or treatment after one month, the rats were slaughtered for histological analysis. A sample of skin was taken from the dissection and preserved in 10% formalin before being processed automatically in a tissue processor as per standard laboratory protocol.[43] The processed tissue was embedded in paraffin wax, sectioned using a microtome at a thickness of 4 µg and stained according to standard protocol using haematoxylin and eosin stain. Stained slides were examined under an optical microscope (under 100x) and digital slide micrographs were captured.[44] The histopathological analysis was carried out for the control group, tumor-induced group and tumor-induced group after treatment with FG4.

RESULTS AND DISCUSSION

Critical Micelle Concentration

The CMC value of the non-ionic copolymer surfactant used (Pluronic F-127) was found to be 4 mg/mL in methanol, 4 mg/mL in acetone and 5 mg/mL in acetonitrile as shown in Figure 1. It can be described that the increase in dye absorbance in Figure 1 indicates that the system's micelles were produced as a result of the dye solubilization by integration into the micellar phase. On the other hand, a heterogeneous mixture with a low absorbance water phase was observed below the CMC due to the insolubility of the dye used. However, the increase in the concentration of polymer in the system resulted in an improvement in the absorbance value as observed here. It is apparent from Figure 1 that the beginning of micellization was indicated by the sharp inflection in absorbance of solubilized dye and non-ionic copolymer surfactant in micelles. Pluronic has an amphiphilic structure because of the hydrophilic ethylene oxide and the hydrophobic propylene oxide.[45] The Pluronic F-127 consists of ethylene oxide (70%) and propylene oxide (30%) by weight and has chain lengths of 200.45 and 65.17, respectively.[46] It has been described that the increase in the chain length of hydrophobic molecules could lead to an elevation in the surfactant's net hydrophobicity, which would encourage the separation of the propylene oxide chains inside micelle core, resulting in a lower CMC.[47] On the other hand, an increase in ethylene oxide chain length could increase the likelihood that propylene oxide will interact with ethylene oxide in the micelle core. Thus, it is expected that increasing the length of the hydrophilic ethylene oxide chain will result in an increase in the CMC value. Moreover, the Pluronic F-127's low CMC value demonstrated could provide better kinetic stability of the tested polymer during dilution with physiological fluid.[24] In addition, the amphiphilic block copolymer stability and hydrophobicity increase with increasing chain length of hydrophobic components.[45] It is well known that stability could be higher with low CMC value,[48] hence the surfactant with low molecular weight was used in the current investigation.

The thin film hydration method was chosen for developing fluorouracil loaded micelles as the selected copolymers can easily dissolve in volatile and water-miscible solvents tested.[48] Indeed, these nanocarrier systems are considered as a viable substitute for traditional topical preparations due to many advantages.[49] In addition, they possess intriguing benefits that render them valuable in the realm of drug delivery systems including elevated drug encapsulation and loading capability. During the process of preparation, the equilibrium between the drug and polymeric micelles in aqueous media was maintained. Various polymeric micelles formulations (FM1-FM12) were fabricated

using Pluronic F-127 as non-ionic copolymer surfactant and fluorouracil as the active pharmaceutical ingredient. The composition details are outlined in Table 1. The polymeric micelles were formulated by varying the drug (50-200 mg), the three solvent systems (methanol, acetonitrile and acetone) with corresponding CMC levels.

Characterization of Fluorouracil-loaded Polymeric Micelles

Entrapment Efficiency

The entrapment efficiency of fluorouracil in the prepared micelles was found to be in the range of 35 to 95% (Table 3). In case of formulations FM1-FM4, when the ratio of fluorouracil to Pluronic F-127 was increased in methanol, there was an increase in entrapment efficiency (54-95%). Similarly, FM5-FM8 showed an increase in entrapment efficiency with acetone with a range of 35-91% and FM9-FM12 with acetonitrile about 51-92%. Further, the increase in the drug: polymer ratio did not yield higher entrapment efficiency, but rather declined probably due to precipitation in micellar suspension. The use of methanol as an organic solvent in formulations (FM1-FM4) shows higher entrapment efficiency as compared to other formulations. This is probably because the type of organic solvent can affect the drug and polymer ratio used in polymeric micelles. Better entrapment efficiency may also be due to the drug's improved interaction with the copolymer and methanol, as opposed to acetonitrile and acetone.

Particle Size Characterization

The two most important factors for topical drug administration are particle size and the uniform size distribution of the drug delivery mechanism.[50,51] The observed particle size of fluorouracil loaded polymeric micelles (FM1-FM12) was shown in Table 3. It is evident that the size of micelles varied in the range of 69.65-159.12 nm while the PDI values ranged between 0.108-0.265. The smaller size (70-160 nm) of prepared nanocarriers could be linked to the low molecular weight of the polymer used. The variation observed in the particle size of prepared polymeric micelles is limited to the ratio of drug to polymer weight (as the copolymer used is the same) as well as the organic solvent. The observed outcomes demonstrated that the drug and polymer concentrations were important factors influencing particle size. The mean particle size of produced fluorouracil-loaded polymeric micelles utilizing methanol (FM1-FM4, 69.65-127.21 nm) and acetone (FM5-FM8, 89.21-98.62 nm) was comparable, however, the particles using acetonitrile (FM9-FM12, 127.28-159.12 nm) were relatively large. The exact reasons for this observation need to be assessed in detail. In micelles prepared using methanol and acetonitrile, when the drug: polymer weight ratio was increased it led to the precipitation in micelles and decreased particle size of the micelles. Additionally, there was no relation noticed between drug loading and particle size of the prepared carriers. The particle size (69.65 nm) and PDI (0.108) of formulation FM4 are shown in Figure 2. In fact, formulation (FM4) was shown to be optimal for topical distribution due to its low particle size range and PDI value suggesting narrow and monodisperse pattern.[52]

Differential Scanning Calorimetry

DSC was performed to study the binding strength of fluorouracil in the micelles. The thermal image of fluorouracil shown a prominent endothermic peak around 282°C as shown in Figure 3, while the composite mixture had an heat absorbing peak of Pluronic F-127 at 54.31°C and 260°C (drug). In case of drug loaded micelles (FM4), endothermic peaks were shifted and were found at 162.51°C for fluorouracil. This is probably because of the physical association between the non-ionic copolymer surfactant

Table 3: Characterization of fluorouracil-loaded polymeric micelles.

Code	Micelle size (nm)	Polydispersity index	% Entrapment efficiency	% Drug release	Best fit model	r^2 value
FM1	107.72	0.238	53.72±0.17	65.25±0.46%	Higuchi	0.9941
FM2	127.21	0.235	57.82±0.21	48.47±0.75%	Higuchi	0.9827
FM3	124.55	0.234	78.54±0.34	87.76±0.53%	Higuchi	0.9880
FM4	69.65	0.108	94.82±0.91	97.82±0.36%	Higuchi	0.9988
FM5	98.62	0.265	34.65±0.25	41.22±0.38%	Higuchi	0.9952
FM6	92.23	0.262	78.76±0.45	81.85±0.86%	Higuchi	0.9849
FM7	94.27	0.112	81.98±0.47	65.93±0.25%	Higuchi	0.9972
FM8	89.21	0.151	91.16±0.91	92.41±0.85%	Higuchi	0.9825
FM9	138.32	0.135	51.23±0.73	63.75±0.75%	Higuchi	0.9862
FM10	149.16	0.262	89.55±0.88	87.56±0.36%	Higuchi	0.9918
FM11	159.12	0.253	54.84±0.87	65.55±0.92%	Higuchi	0.9873
FM12	127.28	0.233	92.21±0.72	89.72±0.76%	Higuchi	0.9813

and the fluorouracil after loading in micelles as reported in the literature.[53]

In vitro Release

The *in vitro* drug release profiles, as illustrated in Figure 4, were used to calculate the cumulative drug permeation (in percentage) for 10-hr periods. The release profile indicates a sustained release of fluorouracil from the prepared micelles, which is essential for topical skin delivery as mentioned in an earlier study.[54] The percentage of drug release at the end of 10 hr in each formulation is depicted in Table 3. It is apparent from the Table/Figure that the percentage varied between 41 to 98%. Due to their high entrapment efficiency and minute particle size, FM4 had the highest cumulative drug release percentage (97.82±0.36%). However, formulation FM5 with the lowest entrapment efficiency showed low drug release (41.22±0.38%). The drug to copolymer weight ratio and the kind of volatile solvent utilised had a minor impact on the drug release variability, which is probably related to the particle size of the micelles. The results of release data were assessed by different kinetic models.[55] The high regression values noticed in Table 3 indicate all formulations fit into the Higuchi model, signifying that the fluorouracil release from the polymeric micelles is proportionate to the square root of time and the diffusion processes regulate the release of the drug through the polymeric membrane.[56] Based on the highest entrapment efficiency and greater drug release, the formulation FM4 was chosen as the optimal formulation and was further developed into a gel formulation.

Transmission Electron Microscopy

The photomicrograph image observed with transmission electron microscopy of Formulation (FM4) indicated the spherical shaped particles with homogenous distribution as shown in Figure 5. The size of micelles was in the nano range and is suitable size for skin drug delivery. The particles size observed here is similar with the data noticed with Zetasizer.

Preparation of Fluorouracil Loaded Micellar Gel

The selected formulation (FM4) was modified into a topical micellar gel using carbopol 940 as a gelling system with the objective to achieve steady drug release of drug from the prepared polymeric micelles. Carbopol was preferred as the gelling agent in this study, because of its excellent buffering capacity and exquisite rheological qualities ideal for cutaneous application.[57,58] The topical gel was formulated by dispersion method and five formulations (FG1-FG5) were prepared according to the composition described in Table 2.

Characterization of Fluorouracil Loaded Micellar Topical Gel

Physical Properties

Visual examination was used to assess the developed gel's color, clarity, spreadability and homogeneity. The FG1-FG5 gels were found transparent and clear, with no phase separation (homogenous in nature) or contamination. pH of topical formulation is a significant factor for its application on the skin surface, as maintaining the ideal pH of the micellar gel formulation may prevent skin irritation and promote percutaneous absorption.[59] Moreover, for a successful topical gel composition, the pH of prepared gel should be necessary to maintain the consistency and stability of the gel. The pH of FG1-FG5 gels was found in the range of 6.7- 6.9 (Table 4), which is ideal for skin application as described in the literature.[59] Spreadability and viscosity have a close relationship and both greatly influence a gel formulation's potency and efficacy.[60] The stickiness of the gel was used to evaluate the formulations' spreadability and it was discovered that they were easily spreadable. The rheology of the topical formulations is of utmost importance, as it affects the quantity and rate at which the drug is released. The viscosity of topical gels was found to be 3871-5383 cP, while the drug content varied between 50-79% (Table 4). The highest amount of drug was found in FG4, followed by FG5 and FG2. Moderately low drug content was noticed with FG1 and FG5 (Table 4). As the drug content was higher in FG4, this gel was selected for further studies.

Skin Permeation Study

Ex vivo permeation studies are typically carried out in order to forecast the drug diffusion through and into the epidermal barriers while in real life use. The permeation of micellar gel into the skin target is of utmost importance to improve the clinical efficacy of the developed formulation. In general, a number of factors affect the drug's ability to diffuse through biological barriers, such as its physicochemical features, the physiological characteristics of the barrier (such as the membrane's composition and thickness) and the transport pathway that the carrier can permeate.[61] Literature indicates that porcine skin's follicular density and stratum corneum thickness are most similar to those of human skin.[62] Numerous *ex vivo* investigations on human skin have also shown a strong association with *in vivo* investigations on pigs. In Figure 6, the *ex vivo* skin permeation profile of FG4 has been compared with the plot of market preparation (Flonida 1% cream). Both formulations tested exhibited a comparable transdermal permeation profile. The steady permeation noticed in Figure 6 (72.50% in 10 hr) could be linked to the higher drug in micellar Gel Formulation used (FG4). Moreover, the sustained permeation from the formulation indicates that the gel matrix used here is suitable for the developed formulation.

In vivo Skin Irritation

To guarantee skin safety during clinical application, it is imperative to assess the degree of skin irritation caused by developed polymeric micellar gel.[63] Typically, the substances used in skin irritation tests are categorized into three levels: non-irritating, slightly irritating and severely irritating. The skin irritation test of the FG4 micellar gel formulation showed a skin irritation score of less than 2. No apparent redness, pimple, erythema, etc. was seen on the skin of rats after three days of application. Overall, the FG4 micellar gel formulation of topical gel was free from skin irritation.

Stability

The selected Gel Formulation (FG4) was assessed for its stability by assessing colour, phase separation, homogeneity, clarity and drug content.[35] The observed data suggests no significant variation in the physical properties assessed. The drug content at zero days and after the third month was comparable, which suggests no chemical degradation of the product during the storage period. In general, the amphiphilic block copolymer-produced micelles retained their integrity and exhibited greater stability.[64]

Cell Line Study

It is crucial to assess the cytotoxicity of the developed polymeric micelles containing Gel (FG4) before considering their potential application in skin cancer. The MTT assay was used to assess fluorouracil's anti-non-melanoma skin cancer activity. Micellar gel was applied in different doses to non-melanoma skin cells, ranging from 25 to 200 µg/mL. The results of the cell viability study are presented in Figure 7. It can be seen that the FG4 shown dose-dependent increased the cell death of the cell line. Cell death was 31.5±3.88% after treatment with 25 µg/mL, which increases with an increase in drug concentration. GraphPad determined IC_{50} of prepared micelles with 5-fluorouracil against cells was found to be 15.56 µg/mL.

Anti-Cancer Activity

After DMBA/TPA application, the weight of rats of different groups was determined for both the control and tumor-induced groups and presented in Table 5. The mean weight of rats in both groups increased with duration. However, a significant difference in rat's weight (p<0.0001) was noticed at the end of 6 weeks, with higher weight in tumor induced group, which is in agreement with the literature.[41]

Application of DMBA/TPA on rats for 6 weeks leads to various changes in the skin like cutaneous edema. The tumor-induced group had the greatest number of lesions (Figure 8), whereas the control group had no tumors. A small-sized, pinkish-white growth and hair loss were noticed at the location where DMBA/TPA was applied (Figure 8), indicating the tumor induction as described in the literature.[42]

Following the confirmation of tumor induction (after 6 weeks of carcinogen application), the skin area was treated with the developed Fluorouracil-loaded micellar Gel (FG4) daily for 1 month. The area and volume of the tumor were measured before the initiation of treatment and after 1 month by the caliper method described earlier.[41] The average area and volume before treatment were 8.46±1.22 mm and 268.52±36.05 mm^3, which reduced to 5.13±0.76 and 55.12±7.22 mm^3, respectively. Indeed, the treatment with fluorouracil-loaded micellar gel for 1 month resulted in a reduction in tumor burden as observed with the area and volume of tumors measured. Overall, the significant reduction in the tumor size signifies the importance of the developed nano drug loaded polymeric micelles.

Histopathological Analysis

Histopathological examination was performed on the skin treated with the fluorouracil-loaded micellar gel formulation and the untreated control group. The micrographs captured are depicted in Figure 9. Histopathological analysis of a skin biopsy shows intact epidermis and dermis (black arrow) as noticed with control (Figure 9A). This normal skin architecture provides a baseline for comparison and shows undisturbed tissue morphology. In non-melanoma cancer-induced animals (Test), skin biopsy shows poorly differentiated epidermal and dermal cells (black arrow, Figure 9B). This abnormal cellular morphology indicates aggressive and unregulated cellular replication typical of non-melanoma cancer. The skin biopsy of the same test group animals after treatment (Treated) shows a well-differentiated epithelial and dermal layer of cells (black arrow, Figure 9C), indicating restoration of tissue architecture signifies the therapeutic efficacy of the formulation. Furthermore, the treatment not only targeted cancerous cells but also promoted cellular differentiation, suggesting the therapeutic potential for repair or regenerative effects on the damaged tissue.

A: hematoxylin-eosin stain, ×100; control, B: hematoxylin-eosin stain, ×100; Test and C: hematoxylin-eosin stain, ×100; treated non-melanoma cancer.

RESULTS

Critical Micelle Concentration

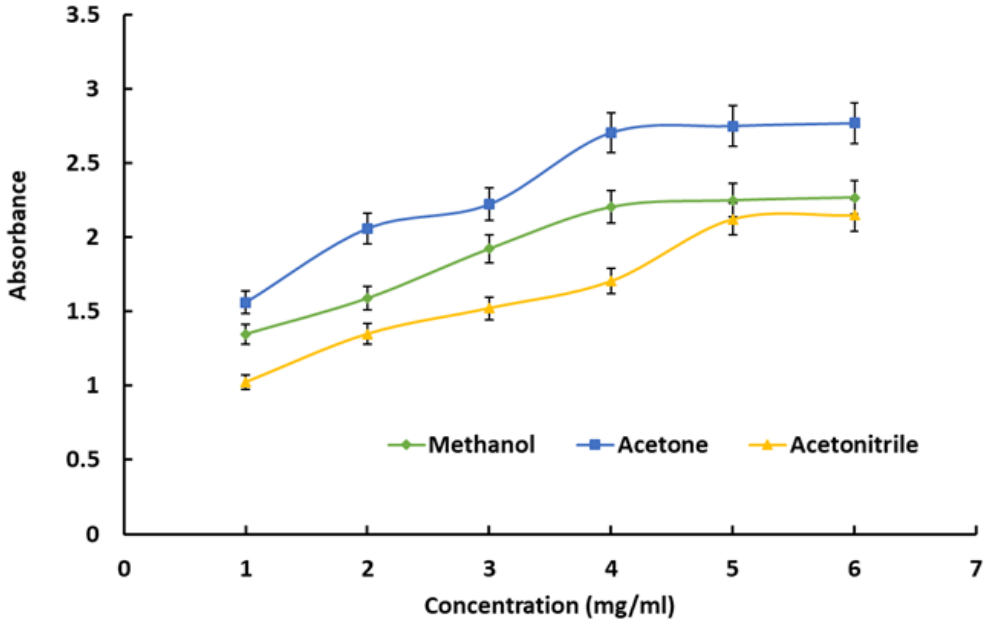

Figure 1: Critical micellar concentration determination of Pluronic F-127 using Sudan III dye.

Characterization of Fluorouracil-loaded Polymeric Micelles

Entrapment Efficiency

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 69.65	Peak 1:	77.45	100.0	25.43
PdI: 0.108	Peak 2:	0.000	0.0	0.000
Intercept: 0.966	Peak 3:	0.000	0.0	0.000
Result quality : Good				

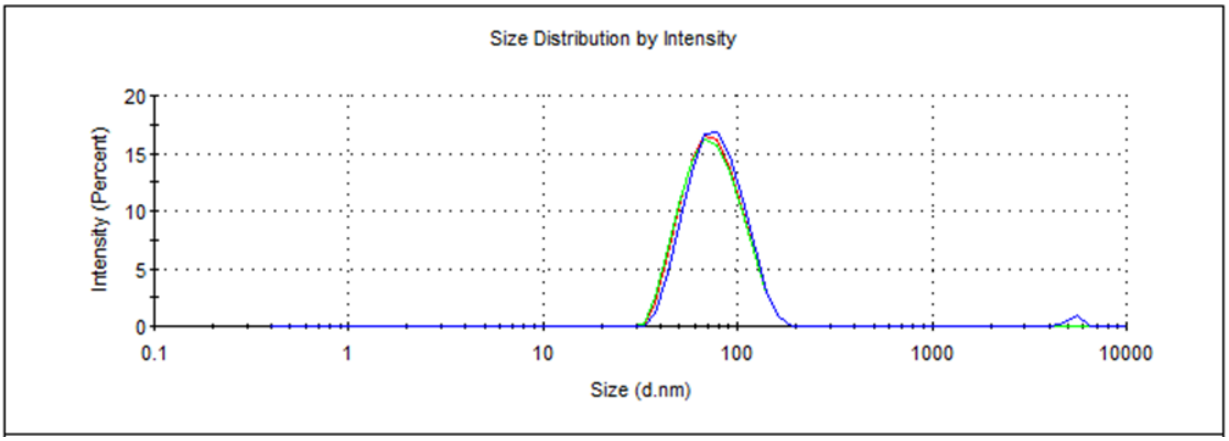

Figure 2: Particle size distribution and polydispersity index of selected polymeric micelles (FM4).

Particle Size Characterization

Differential Scanning Calorimetry

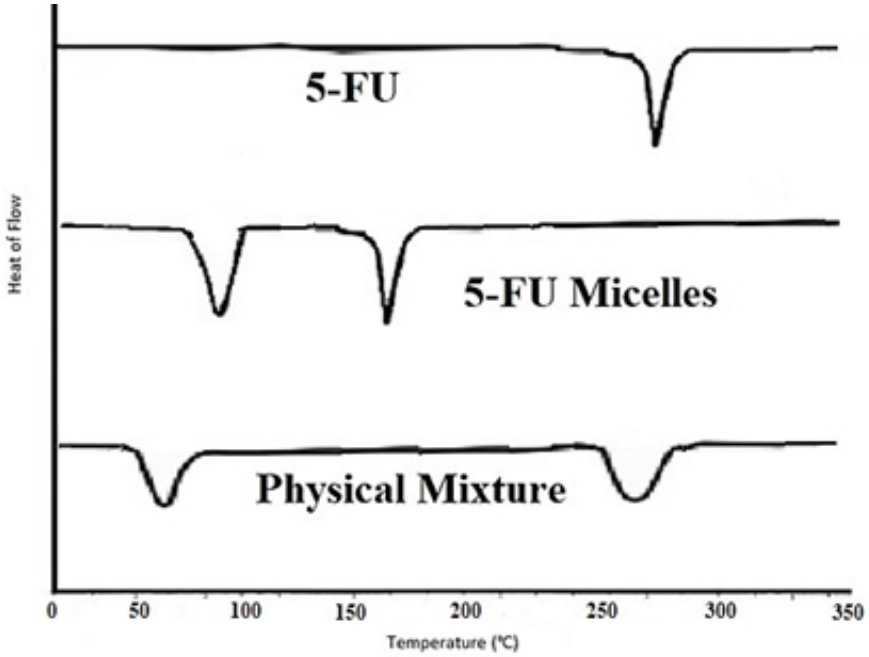

Figure 3: DSC thermogram of fluorouracil, drug and polymer mixture and polymeric micelles (FM4).

In vitro Release

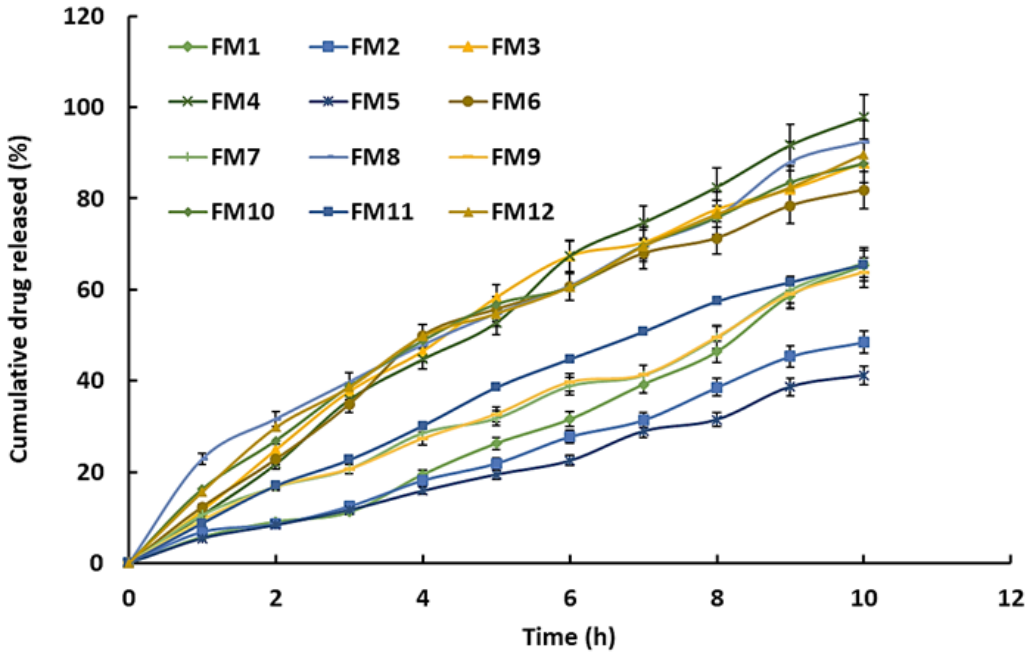

Figure 4: Comparative *in vitro* drug permeation profiles of fluorouracil from developed polymeric micelles (FM1-FM12) using Franz diffusion cell.

Transmission Electron Microscopy

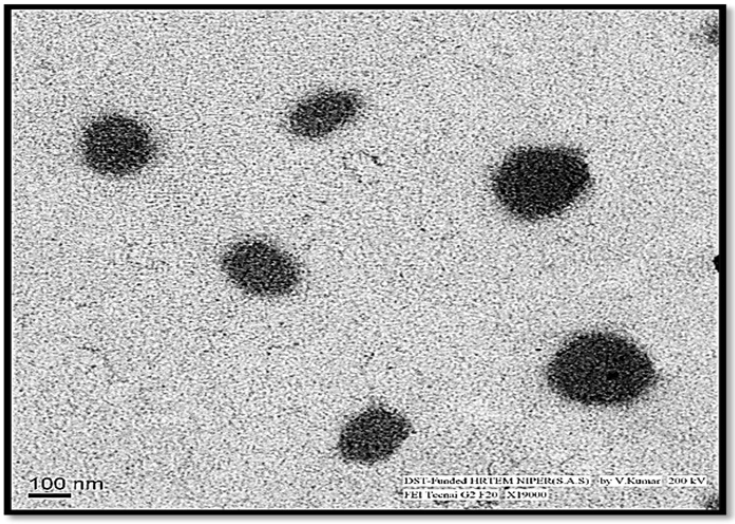

Figure 5: Transmission electron microscopy image of optimized fluorouracil polymeric micelles (FM4).

Characterization of Fluorouracil Loaded Micellar Topical Gel

Physical Properties

Table 4: Characterization of fluorouracil loaded micellar gel (FG1-FG5).

Formulation Code	Drug Content (%)	pH of gel	Viscosity (cP)
FG1	57.27±0.65	6.7	3871±55
FG2	62.85±0.74	6.9	4384±76
FG3	49.48±0.82	6.7	4876±104
FG4	78.64±0.34	6.8	5081±95
FG5	67.23±0.46	6.8	5383±112

Skin Permeation Study

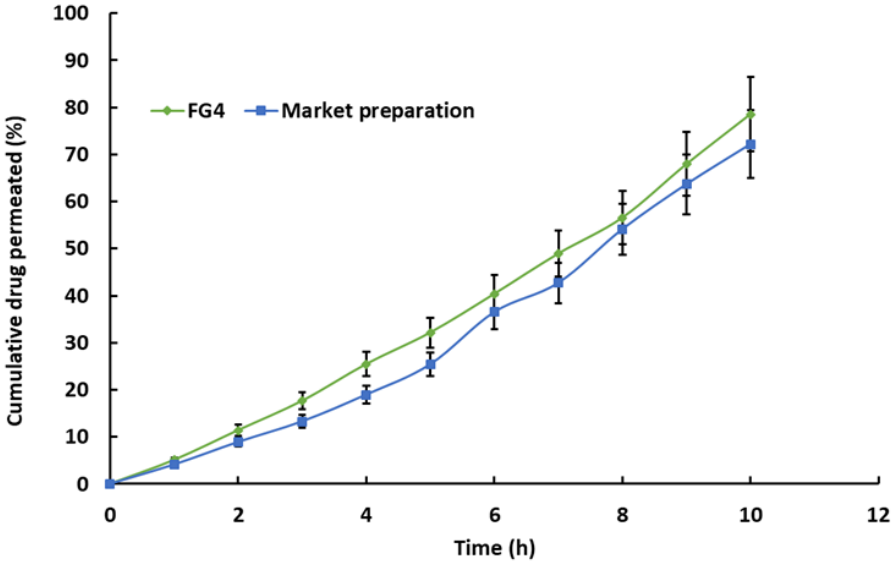

Figure 6: Comparative *ex vivo* permeation profiles of fluorouracil loaded polymeric micellar gel (FG4) and market preparation (Flonida 1% cream).

Cell Line Study

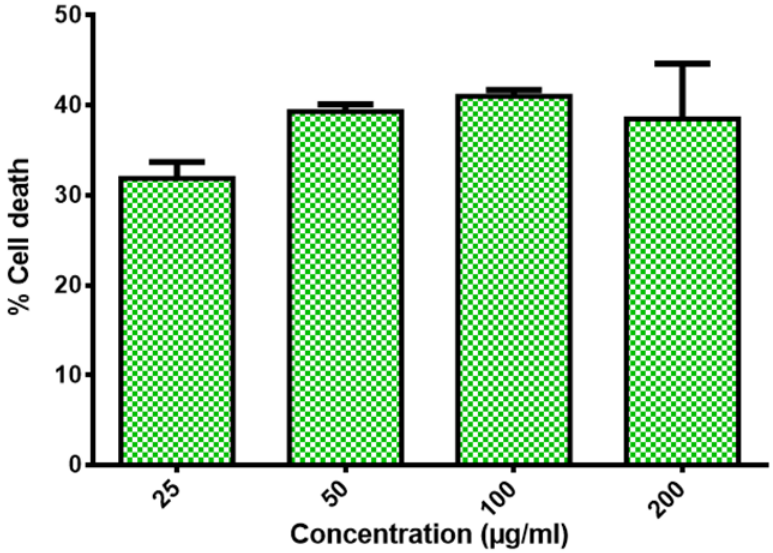

Figure 7: MTT assay results showing cell viability of developed fluorouracil loaded polymeric micellar gel (FG4).

Anti-Cancer Activity

Table 5: Weight of rats observed during tumor induction study.

Groups	Week 0 (g)	Week 2 (g)	Week 4 (g)	Week 6 (g)
Group A (Control)	223.63±3.51	240.92±1.87	252.57±3.15	267.92±2.38
Group B (Tumor-induced)	221.24±3.11	243.65±2.31	266.44±7.54	299.52±6.21

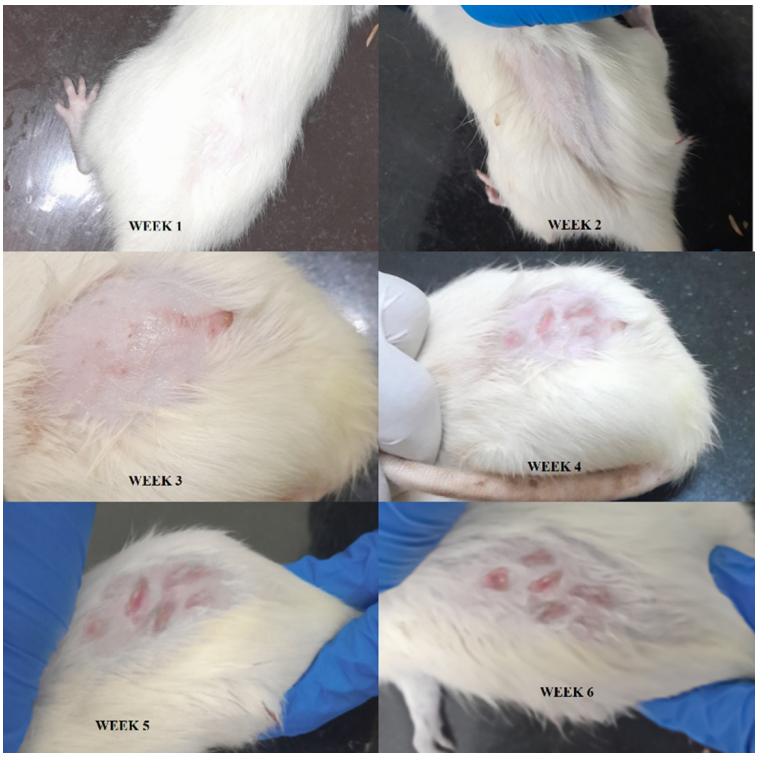

Figure 8: Tumor induction in albino rats.

Histopathological Analysis

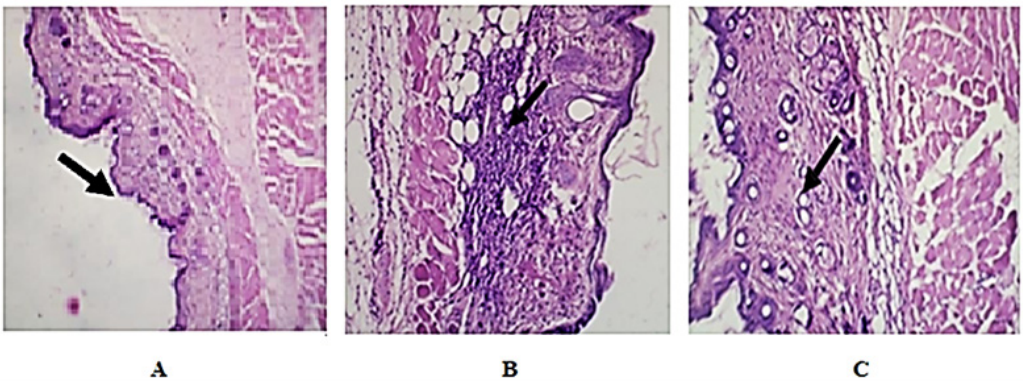

Figure 9: Histopathological examination showing the comparison of skin treated with fluorouracil-loaded micellar gel and the untreated control group.

CONCLUSION

Topical formulations for the treatment of skin cancer are less toxic to the body and yield better results. Topical administration of fluorouracil was chosen due to several limitations associated with its oral route, including bloody stools, chest pain and convulsions. The developed fluorouracil topical gel loaded with polymeric micelle exhibited more efficiency and promised to be a superior drug delivery system. Indeed, the prepared formulations demonstrated the ability to deliver drugs topically to the skin. It can minimize the systemic side effects associated with fluorouracil oral administration and addresses the limitations of the marketed preparation without endangering patient safety or compliance. Sustained drug release (up to 10 hr) without causing skin irritation or toxicity was demonstrated by the new formulation. Overall, the Pluronic F-127 micelles exhibit promise as hydrophobic drug carriers for the skin, perhaps aiding in the treatment of non-melanoma skin cancer.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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ABBREVIATIONS

DMBA: 7-12-Dimethyl-1-benz (a) Anthracence; **TPA:** 12-O-Tetradecanoy-1-Phorbol-13-Acetate; **PDI:** Polydispersity Index; **C:** Celsius; **CMC:** Critical Micelle Concentration; **µg:** Microgram; **µM:** Micrometer; **PBS:** Phosphate Buffer Saline; **mg:** Milligram; **BCS:** Biopharmaceutical Classification System.

ETHICAL APPROVAL

The ethical approval for the experiment was procured from the institutional ethical committee (1355/PO/Re/S/10/CPCSEA) with protocol no. MMCP-IAEC-102.

AUTHOR CONTRIBUTION

Conceptualization, Navneet Mehan, Vipin Saini, Manish Kumar, Sheetal Devi; Data Curation, Navneet Mehan, Vipin Saini, Manish Kumar, Manoj Goyal, Sheetal Devi, Shinu Pottathil, Shery Jacob, Anroop B Nair; Formal Analysis, Navneet Mehan, Vipin Saini, Manish Kumar, Sheetal Devi, Anroop B Nair; Investigation, Navneet Mehan, Vipin Saini, Manish Kumar, Manoj Goyal, Sheetal Devi, Shinu Pottathil, Shery Jacob, Anroop B Nair; Methodology, Navneet Mehan, Vipin Saini, Manish Kumar, Manoj Goyal, Sheetal Devi, Shinu Pottathil, Shery Jacob, Anroop B Nair; Writing-Original Draft Preparation, Navneet Mehan, Manoj Goyal, Sheetal Devi, Shinu Pottathil, Shery Jacob, Writing-Review and Editing, Vipin Saini, Manish Kumar, Anroop B Nair. All authors have read and agreed to the published version of the manuscript.

SUMMARY

We find the anti-cancer drug delivery systm by using Fluorourail loaded micelles. The results was found to be very precise and accurate, also this formulation was more convenient for application. Therefore, it could be useful for the treatment of non-melanoma skin cancer treatment.

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