The Sun Pharma Science Foundation Science ScholarAwards-2023

(Pharmaceutical Sciences category)

Title:

Vesicular Drug Delivery System for Skin Cancer

Introduction

As per WHO Skin cancer appears to be one of the most prominent diseases, affecting a wide variety of population. There has been a significant increase in prevalence rate through the latter half of the twentieth century. More than 90% of all skin cancer-related mortality is caused by malignant melanoma a mthest complicated type of skin cancer. As a result, most skin malignancies may be detected in their early stages. Every year over 1.5 million new cases of skin cancer are discovered, and the prevalence of skin cancer is on the rise. Basal cell carcinoma accounts for around 80% of skin malignancies, whereas squamous cell carcinoma accounts for 15%. Even though genetic predisposition and skin pigmentation are the most significant risk factors. Cancer has a multimodal cause, with genetic changes, environmental influences, and social factors all playing a role.

Its origin is linked to several predisposing variables, including skin type, age, exposure to sunlight, poor pigmentation potential, hereditary diseases (such as xeroderma pigmentosa, albinism, and others), and immunocompromise, among others. Furthermore, skin cancer is the most common malignancy among organ transplant patients. Cancer develops each year when DNA, the chemical present in cells that encodes genetic information, is broken and the body is unable to repair it. These injured cells start to multiply and expand at an uncontrollable rate. Skin cancer begins when this happens on the skin. A tumor develops when the injured cells proliferate. A ttumoris frequently apparent because skin cancer grows in the epidermis, the outer layers of the skin. As a result, most skin malignancies may be detected in their early stages.

Various conventional therapies, such as biopsy surgery, Mohs surgery, electro surgery, and cryosurgery, are some of the main treatments for skin cancer treatment. Other treatments include laser therapy, radiation therapy, photodynamic therapy, and chemotherapy. Topical fluorouracil and Drug A, orally administered vismodegib, and other therapeutic agents such as cisplatin, Ipilimumab, doxorubicin, etoposide, topotecan, interleukin-2 (IL-2) interferon 2b, dacarbazine, MEK inhibitors, BRAF inhibitors, and others are used for chemotherapeutical management of skin cancers. Current treatments have several drawbacks, including a lack of cell selectivity, frequent relapses, significant toxicity, and higher costs. The American Cancer

Society found that after chemotherapy, some people found a recurrencof e cancer.

Transethosomes are ideal for delivery as they are biocompatible, totally biodegradable, non-toxic, flexible, and non-immunogenic. Drug A works by stimulating the immune system to release several chemicals called cytokines, which are important in destroying cancer cells. Also, combination with β-blocker Drug B, commonly used to manage cardiovascular disorders such as hypertension and heart failure, showed promising activity in preventing chemical carcinogens and UV-induced skin carcinogenesis in vitro and in vivo.

Nanomedicine for skin cancer has already become a current scientific practice, and it has demonstrated a major evolution in the treatment of skin tumors, including aggressive and invasive tumors. The problem of resistance to chemotherapeutic agents is perhaps one of the greatest challenges in clinical medicine. As a result, the Nano vesicular delivery system has benefits such as specific passive targeting of tumor tissues, increased effectiveness and therapeutic index of medicinal molecules, increased stability by encapsulation, and reduced side effects of encapsulated drugs. It also exhibits a site avoidance effect, enhances drug molecule pharmacokinetic characteristics (lower elimination, increased circulation life durations), provides flexibility in coupling with site-specific ligands to accomplish active targeting, and aids in reducing harmful drug exposure to healthy tissue. The developed vesicular system was in liquid form, thus having less viscosity which could not be applied topically. Therefore, it is incorporated into selected hydrogel such as Carbopol 934 shown to be the most feasible, efficient, and effective method of distribution. It also leads to better therapeutic release. In the field of dermatology, a dual controlled-release system is shown in a formulation incorporated into hybrid rid gel.

The 5-FU gel is sold on the market and has been shown to be quite effective in treating skin cancer. It is a BCS-III drug candidate, which limits the effectiveness of the medicate the oral route, that prevent it from being an effective chemotherapy agent. These drawbacks include quick metabolism, a short half-life (15 min), poor bioavailability, rapid first pass first-pass and, most significantly, the drug's non-specificity to cancerous cells, which results in chemoresistance when 5-FU is administered consistently. Also, a number of several claimed that administering 5-FU intravenously resulted in hypersensitivity responses and cytotoxic consequences as compared to oral treatment. It has been discovered that transfersomal the 5-FU with a Carbopol base has higher penetration and causes no discomfort. According to prior studies, the beta-blocker carvedilol, which is often used to treat cardiovascular diseases including hypertension and heart failure, has demonstrated potential effectiveness in lowering chemical carcinogens and UV-induced skin carcinogenesis in vitro and in vivo. In addition to

decreasing UV-induced oxidative stress, DNA damage, inflammation, and carcinogenic signaling pathways, carvingilol possesses several other anticancer mechanisms as well. Also, a population-based cohort study of 6771 individuals found that long-term carvedilol use was associated with a lower incidence of several types of cancer, suggesting carvedilol's potential role in cancer prevention.

Topical administration is an important alternative method for the prevention of skin cancer since it is an easy and well-liked self-treatment strategy and improves the possibility that the medication will reach the areas of injury. Because of its high lipophilicity, low molecular weight, and favorable logarithmic partition coefficient, the drug carvedilol can be administered through the skin (log p value of 3.8). As acetone is insoluble in water, it is used as a carrier and a skin penetration enhancer for topical administration of carvedilol in animal models. Although acetone is widely used in animal skin cancer studies as a carrier, it is not recommended to use it on humans since it severely dehydrates and dries up the skin. Moreover, using acetone increases the risk of unintentional systemic absorption of topical drugs since it compromises the skin's natural barrier function.

Hence, we suggested delivering 5-FU and CVD topically to target the epidermal and dermal layers for greater retention and accumulation of treatments while lowering drug clearance. We propose unique dual-drug (CVD and 5-FU)-loaded transethosomes for better chemotherapy based on all available scientific data. By encasing the pharmaceuticals in lipidic TEs, the main pharmacological properties of the medications, such as solubility and permeability, were enhanced. Moreover, the combination's synergistic effects enhance chemotherapeutic effectiveness while eliminating chemoresistance. Importantly, these nanocarriers were added to the gel to facilitate topical medication delivery. The combination of CVD and 5-FU enhanced more than just the pharmacological properties; it also substantially increased bioavailability and decreased the dose of CVD and 5-FU needed to slow tumour growth. To the best of our understanding, this is the first study of its kind to combine 5-FU and CVD, which are then combined with a transethosomal carrier to target the epidermal layer. This study improved the sensitivity of 5-FU and CVD for successful therapy, demonstrating the effectiveness of these two medications in treating skin cancer. By improving medication distribution to the skin layer through the use of a transethosomal carrier, conventional chemotherapy's systemic adverse effects may be lessened. To ascertain the long-term safeguards and efficacy of this therapeutic strategy, more research is required.

Aim

Formulation And Evaluation of Vesicular Drug Delivery System of Anti-cancer Drugs for Topical Management of Skin Cancer

Objectives

1. **Pre-formulation Studies**
    - Drug and drug-Excipient interaction
2. **Formulation and evaluation of Vesicles**
    - Morphology of Vesicles
    - Stability studies of vesicles,
    - Biological evaluation of vesicles
        - Dermal Kinetics
        - Pre-clinical efficacy Studies
3. **Formulation and evaluation of dosage form for vesicles**
    - Formulation of hydrogel
    - Stability studies,
    - Biological evaluation of gel vesicles
4. **Application of Statistical treatment by various statistical methods**

Material and Methods

Materials: -

All drugs and excipients were got as a gift sample for the research project.

Table 1a ***List of Procurements of API***

API	Name of Industry
5-Flurouracil (5-FU)	1. Yarrow pharma Supplied by Carbanio 2. Alfa Aesar supplied by Thermo fisher Scientific as Gift Samples
Carvedilol (CAR)	Yarrow pharma Supplied by Carbanio

Table 1b List of Different Type of Membrane Filter

Membrane Filter by Globe scientific as Gift Samples

1. Syringe Filter, Nylon Membrane, 0.22µm Porosity, 30mm Diameter, PP Housing, Non-sterile
2. Syringe Filter, PTFE Hydrophobic Membrane, 0.22µm Porosity, 30mm Diameter, PP Housing, Non-sterile
3. Syringe Filter, PTFE Hydrophilic Membrane, 0.22µm Porosity, 30mm Diameter, PP Housing, Non-sterile

Table 1c List of different Excipients

Sr. No.	List of Excipients as Gift Samples	Name of Industry
1	Lipova E80	Lipoid Gmbh
2	Lipoid S 100	Lipoid Gmbh
3	Cholesterol	VAV Lipids P. Ltd.
4	Phospholipon 90 H	VAV Lipids P. Ltd.
5	KolliPhore P 338 G	BASF India Ltd.
6	Kolliphore P 188 G	BASF India Ltd.
7	Kolliphore RH 40	BASF India Ltd.
8	Kolliphore EL	BASF India Ltd.
9	Kollicoat IR	BASF India Ltd.
10	Kollisolv PG	BASF India Ltd.
11	Transcutol® P	Gattefosse
12	Capryol® 90	Gattefosse
13	Plurol® Diisostearique	Gattefosse

Sr. No.	List of Excipients as Gift Samples	Name of Industry
14	Tween 20	CRODA India P. Ltd.
15	Tween 60	CRODA India P. Ltd.
16	Span 20	CRODA India P. Ltd.
17	Span 60	CRODA India P. Ltd.
18	Span 80	CRODA India P. Ltd.

Methodology

1. **Preformulation studies**
   i. Organoleptic properties of Carvedilol and 5-Flurouracil
      The purchased fisetin drug sample was characterized based on different physical properties such as color, odor and state.
   ii. Solubility studies in aqueous and non-aqueous solvents
   iii. Melting point determination
   iv. Partition coefficient studies.
   v. UV Spectra analysis Preparation of standard curve of drug of both drugs

Preparation of standard stock solution of Carvedilol:

Accurately weighed 10mg of carvedilol pure drug taken in separate 100mL volumetric flask and dissolved with 25mL of methanol and shaken for 10 min and then diluted with methanol up to mark to get 100 µg/mL standard stock solution.

- **Concentration of calibration curve:**

Aliquots of standard stock solution were pipetted out and suitably diluted with methanol to get final concentration of 1-12 µg/mL. The solution was scanned in the spectrum mode from 200nm – 400nm wave length range and sharp peak was obtained at 248nm. Calibration curve was constructed by plotting the absorbance against the concentration and regression equation was computed.

- **UV Spectral Analysis of 5-fluorouracil in Distilled Water:**

Determination of absorption maximum in Distilled Water:

A stock solution of 5-fluorouracil (100µg/ml) was prepared by dissolving 10 mg of drug in water and final volume was made to100ml. A dilution of (10 µg/ml) was kept in cuvette. The solution was scanned in the range of wavelength 200 – 400 nm. The UV spectrum showing λmax was recorded using double beam UV-Visible spectrophotometer.

- **Preparation of Standard Curve of 5-fluorouracil in Water**

A stock solution of 5-fluorouracil (100 µg/ml) was prepared by dissolving 10 mg of drug in Water and final volume was made to 100 ml. The solutions in concentration range of 2- 12 µg/ml were prepared by appropriate dilutions of stock solution. The UV absorbances of these solutions were determined Spectro photometrically at λmax 266nm using double beam UV-Visible spectrophotometer.

vi. Partition coefficient estimation of Carvedilol and 5-Flurouracil

In octanol/water, the partition coefficients of Carvedilol and 5-Flurouracil were calculated. Water and octanol were mixed in graduated tubes (50 ml each). Then, in each flask, 20 mg of Carvedilol and 5-Flurouracil were added, and the mixtures were mechanically shaken for 24 hours. The resultant mixture was then transferred to a separating funnel and left to equilibrate for 6 hours. The quantity of Carvedilol and 5-Flurouracil retained in the octanol was measured using a UV spectrophotometer at 248nm and 266nm, respectively, in the separated octanol and water in separate flasks. For partition coefficient estimate, the following formula was used.

Partition coefficient, PC = (Ct-Ca)/ Ca Where Ca= Ct- Ca

UV spectral analysis Ct is total Carvedilol and 5-Flurouracil concentration taken (20 mg) Ca is total Carvedilol and 5-Flurouracil concentration aqueous phase

vii. High performance liquid chromatography method

The HPLC analysis of Carvedilol and 5-FU was carried out by HPLC system HPLC automated system (Shimadzu and Agilant) attached with variable wavelength detector. having 20 ml loop size.

Chromatographic conditions for Carvedilol and 5-FU API

Table 2 ***List of parameters optimization of chromatography***

Sr.no.	Parameters	Observation
1	**Column**	Column: Kromasil 100-5-C18 Serial no. E187691 4.6 x 100 mm
2	**Mobile Phase**	Mix Buffer & Acetonitrile in the optimised ratio
3	**Wavelength**	254 nm
4	**Flow Rate**	1.0 ml / minute
5	**Injection volume**	20 µl
6	**Run time**	15 minutes
7	**Diluent**	Use Mobile phase as diluent

viii. Differential scanning calorimeter (DSC) analysis

DSC measurement of Carvedilol, 5-Flurouracil drug and Excipients was carried out by using differential scanning calorimeter Instrument Each drug sample (5 mg) was transferred to aluminium DSC pan and sealed to execute the analysis by heating the sample from 30 to 300C with a heating rate of 10 C/min.

ix.In Silico Studies

All the Computational studies presented such as molecular docking, MM-GBSA, and ADME analysis were performed on Docking was performed by AutoDock 4.2.6 program, using the implemented empirical free energy function and the Lamarckian Genetic Algorithm (LGA). Firstly, Ligand preparation was initiated by generating the structure of Carvedilol and 5-Fluorouracil in chem draw tool version 16.0 in cdx format, PubChem CID were redeemed and saved in SDF format. Furthermore, ligand preparations were continued by taking the 3-D structure of all the ligands and were introduced in Pymol software for conversion of 3-D structure from SDF to PDB format.

x.Compatibility study Screening of excipients, Drug –excipients interaction study

xi.HPLC calibration plot with RP-HPLC method development and validation

2. Formulation Of Transethosomal Formulation of CVD-FU based Transethsomes

Previously reported ethanol injection method with some modification the modified ethanol injection injection-sonication method was utilized to prepare CVD-FU-TEs formulations. In brief, a precisely weighted quantity of CVD was transferred into a Beaker, then the amount of Lipoid S100, Tween 80, and ethanol calculated was added (alcoholic phase). To prevent ethanol evaporation, the system was tightly sealed. The aqueous phase was made up of freshly prepared deionized water contain 5-FU and polyvinyl alcohol as a surfactant. The alcoholic phase Injected into Aqueous phase using an intravenous set system with a flow rate at 30±2◦C. The alcoholic phase Injected into Aqueous phase using an intravenous set system with a uniform flow rate. Finally, the size of the developed vesicles was reduced by sonication for 5 min, with 10 s rest between cycles, further pass-through membrane filter PTEE (0.22µm) for size extrusion. Final Formulation was centrifuged to remove free drugs and dilute with aqueous phase stored at 4 ◦C for further study. Method of preparation of transethosomes represented in figure 1.

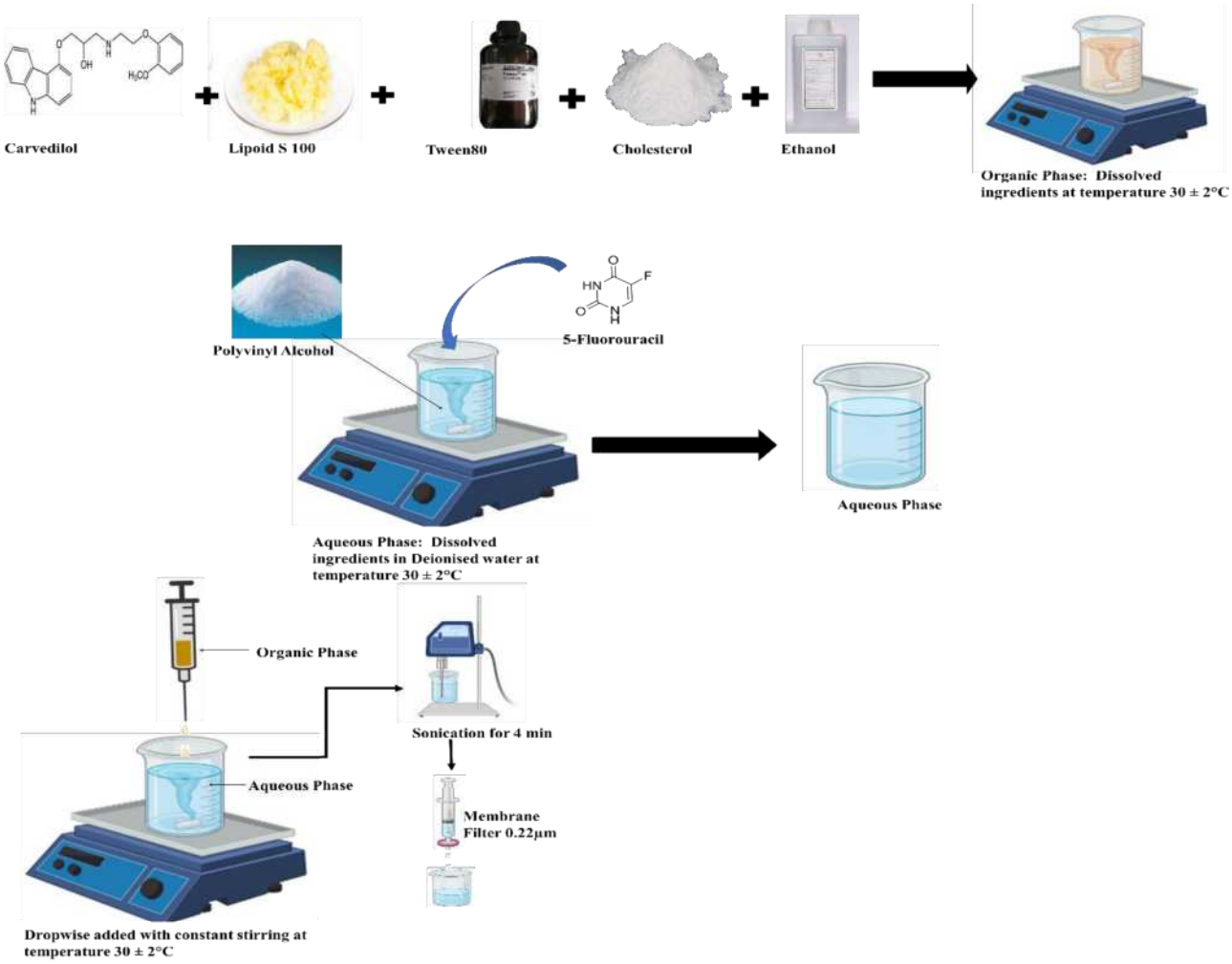

Figure 1 Method of preparation of CVD-FU loaded Transethsomes

3. Optimization Of Transethosomal Formulation by Box Behnken Design

Utilising Design Expert software 11, it was determined how the composition of the TEs affected their physicochemical properties (State-Ease Inc., USA). It produced 17 experimental runs in a Box Benken design matrix, 3D response-surface plots, and statistical analyses. To ascertain the impact of independent variables like phospholipid (Lipoid S100), surfactant (Tween 80), and ethanol concentration, according to the preliminary experiments, here, their effects on dependent variables like vesicle size, zeta potential, encapsulation efficiency of CAR, entrapment efficiency of 5-FU, and others were interpreted at three levels, coded as 1, 0, and + 1 (Abdallah et al., 2022; Aodah et al., 2023; Mishra & Kaur, 2022). Table 1 provides a thorough explanation of dependent and independent variables.

4. Evaluation Of CVD-FU Loaded Transethosomal Formulations

i. Vesicle size distribution and zeta potential

A zetasizer device was used to analyse the size distribution and vesicle size of transethosomes (Nano ZS, Malvern Instruments, UK).

ii. Vesicle morphology SEM, TEM, AFM

On a Field emission Scanning electron microscopy (FESEM) stub, dried FU-CAR-TEs of the optimised formulation were placed. The sample was then coated with a thin layer of gold using a Sputter Coater JFC-1600 while being vacuumed (JEOL, Japan). The samples were run and examined, and a FESEM (S8010, Hitachi, Tokyo, Japan) with an accelerating voltage of 15 kV was used to characterise the morphologies and nanostructures of the TEs samples.

Transmission electron microscopy (TEM) was used to investigate the morphology of transethosomes that had been generated (Tecnai, G20, Philips scientific, the Netherlands). In a nutshell, one drop of the diluted ethosome sample was applied to a copper grid and allowed to dry. After drying, the sample was fixed with phosphotungstic acid, 1% w/v. After that, a TEM was used to inspect the sample on the copper grid and take pictures.

The samples were centrifuged at 7378 g (Sigma Laborzentrifugen, Osterode am Harz, Germany) for 10 and 20 min, for CVD-FU-loaded transethosomes, before being resuspended in water (in half of the previous volume), for the **atomic force microscopy (AFM)** investigation. AFM employs a 1:2 diluted sample that is not pre-treated (Mota et al., 2021). A drop (about 40 L of sample) was applied on a newly split mica surface and left to adsorb for about 30 min. Using a stream of nitrogen to dry the samples, they were then subjected to intermittent analysis (Multimode 8 HR Microscope, produced by Bruker, Billerica, MA, USA).

iii. Entrapment efficiency

The ultraviolet (UV)-visible spectrophotometry technique was used to assess the prepared transethosomal formulation's entrapment efficiency (EE). The resulting transethosomal formulation was briefly centrifuged for 20 minutes at 4 degrees Celsius to separate the transethosomes from the dispersion, then washed three times with distilled water. Also, the free drug contained in the supernatant layer of the centrifuged FU-CVD-TEs dispersion was measured using a UV spectrophotometer (Shimadzu 1800, Japan) at wavelengths of 266 nm and 284 nm for 5-FU and CVD, respectively. Then, (EE) was established utilising equations.

The entrapment of FU-CAR in transethosomes was determined separately by using equation.

$$Percent\ Entrapment\ Efficiency\ (\%EE) = \frac{amount\ of\ drug\ loaded\ in\ TEs}{total\ amount\ of\ drug\ used\ to\ prepare\ TEs} * 100$$

Equation 1

iv.Degree of deformability or permeability measurement

FU-CAR-TEs were subjected to deformability testing utilising modified extrusion facilities. A syringe linked to a membrane filter with a pore size of 0.2 m was used to remove the samples (2 mL), and the extrusion process was carried out by continuously placing a calibration weight of 500 g on top of the syringe. Both prior to and following the experiment, the particle size was assessed, and the deformability index was calculated using the equation.

$$D = J\left[\frac{r_v}{r_p}\right]^2 \qquad \textit{Equation 2}$$

where J is the amount of formulation extruded in 5 minutes; r_v is the particle size following the experiment; and; r_p is the membrane pore size. D is the deformability index of nanovesicles.

v. X-ray diffraction analysis (XRD)

XRD confirms the structural properties of the pure drug and the prepared formulation FU-CVD-TEs. Also used to investigate the amorphous phase of the drug within the formulations. XRD (X-ray diffraction) patterns were determined using PW 1830, a Philips equipped with an XRD commander programme to investigate the crystalline peak of the sample, which results in ensuring the physical state of the sample. Briefly, pure FU, CVD, and FU-CVD-TEs were investigated by the Bruker-AXS D8 Advance diffractometer (Karlsruhe, Germany) with a Cu K radiation source fixed at 40 kV and 100 mA. XRD was performed at a scanning rate of 1 /s over a scanning range of 10–70 degree.

vi. Raman spectroscopy

Using a spectrophotometric system (Thermo-scientific instrument (DxRxi)), outfitted with an OMINICxi-Analysis software, the Raman spectra of the FU-CVD loaded transethosomes were examined. The spectra were collected using a 532 nm laser beam with a 5-100 mW laser intensity. To scan and collect the complete spectrum, a range of 125-4000 cm 1 was used.

xii. Comparative Studies of Formulation

Liposomes were prepared by ethanol injection method by using Lipoid S100, cholesterol. Ethosomes were prepared according to the method of Ethanol Injection. Lipoid S100, Cholesterol, Ethanol. The mixture was hermetically closed, to avoid solvent evaporation, and maintained under stirring at 65°C for 10min. The obtained dispersion was left cooling down, sealed, protected from light and stored at 4°C.

Transferosomes were prepared according to the same procedure, but using a lipid phase having the following composition: lipoid S100 and an edge activator (Tween 80), co-surfactant (PVA) and cholesterol. Transethosomes reproduced by optimization method represented previously. Further Liposomes, ethosomes, transferosomes, transethosomes were comparative evaluation studies as Vesicle size, PDI, Zeta Potential, Percent drug entrapment efficiency. Also, all samples are kept for stability studies.

vii. *In-vitro* release studies

The generated FU-CVD-TEs formulations of the modified ethanol injection technique were tested using a transdermal diffusion cell system (SFDC6, LOGAN Instrument, NJ) outfitted with Franz diffusion cells (Microette Plus, Hanson Research, CA, USA). The vesicle formulations (0.2 ml equivalent to 1.5 mg of LCZ) were applied to the donor compartment using a dialysis membrane (molecular weight cut off 12,000 Da), and a receptor volume of 25 ml of phosphate buffer saline (PBS, pH 7.4) containing 1.5% Tween 80 was maintained at 37 0.5 °C under constant stirring (75 rpm) throughout the experiment. For each experiment, 0.5 mL of the receptor medium was collected at intervals of 1, 2, 3, 4, 6, 8, and 24 hours. The receptor compartment was then immediately replenished with the same volume as a pure receptor vehicle. Prior to HPLC analysis at 254 nm, all samples were filtered via a membrane filter with a 0.45 m pore size. The following equation had been used to determine the 5-FU and CVD release percentages for all formulations, and three replicates had been shown.

$$\text{Cumulative release (\%)} = \text{The amount of each drug at time t} / \text{The total amount of each drug} \times 100$$

xiii. Ex vivo skin permeation study

Fresh abdominal skin of goat was used for skin permeation studies. Skin was obtained from local slaughterhouse. The tissue was then washed with distilled water to remove the mucous and other adhered matrices. Tissues of about 0.2 mm thickness and 3 cm length were mounted on Franz diffusion cell having surface area of 1.79 cm^2 and volume of 25mL. The tissue was stabilised using mixture of methanol and 0.2M phosphate buffer saline (pH 7.4) in the ratio of 1:9 as medium, in both, donor and receptor compartments with magnetic stirring for 30 min. At the end of 30 min, the existing buffers in both the compartments were replaced with fresh medium. The receptor compartment was stirred at 100 rpm. Formulation (1 ml) was kept in donor compartment over the goat skin which acted as diffusion barrier. Study was conducted for 24 h. Samples were withdrawn at fixed intervals and replaced with same amount of fresh media to maintain sink conditions. Sample analysis was carried out using HPLC at 254nm. Each study was carried out in triplicate.

xiv. Skin retention study

Skin was washed with 0.2 M phosphate buffered saline (7.4 pH) 3–4 times to remove the adhered formulation. To confirm the complete removal of any adhering drug, the washings were subjected to HPLC. The absence of drug peak indicated that the skin was free from any adhered drug. Cleaned skin was cut into small pieces and kept in mixture of methanol and 0.2 M phosphate buffer saline (pH 7.4) in the ratio of 1:9 for 24 h to extract out the drug deposited inside the skin. After 24 hr, the medium was sonicated and centrifuged. Supernatant was collected and analysed using HPLC for quantitation of drug. Each study was conducted in triplicate and mean data were recorded.

xv. Haemolysis Study

Hemolysis studies were carried out to observe whether topical administration of nano-formulation causes destruction of RBCs. Three Eppendorf tubes were prepared in which a positive control included triton-X surfactant known to cause haemolysis. A negative control included 0.1N NaOH solution and the third one included the component to be tested. Similar type of arrangement was done for API and for nano-formulation. The contents of tubes were mixed thoroughly. After allowing them to stand, the contents were examined under motic microscope to obtain images.

viii. Stability studies

The stability studies of optimized transethosome and ethosome formulations were carried by analyzing their physical or chemical characteristics during storage. The stability studies were performed in accordance with the ICH guidelines (ICH-Q1A R2). The formulations taken in borosil glass container (USP glass type II) were analyzed for 6 months by keeping in two different storage conditions such as 4±2 ^{0}C and 25±2 ^{0}C/60±5% RH. The following parameters were analyzed for the formulations at specific time period of 0, 1, 3 and 6 months. Sam condition to be followed by gel formulation. Visual observation Transparency, translucency, clarity and homogeneity of the formulations were assessed by visual observations Phase separation study Phase separation was also analyzed by visual observation. Vesicle size and polydispersity index: Photon correlation spectroscopy (NanoZS90, Malvern instrument Corp., UK) was utilized for determining the vesicle size and polydispersity index of developed transethosomes and ethosomal formulations. Encapsulation efficiency Ultracentrifugation method was used for estimation of encapsulation efficiency of developed transethosomal and ethosomal formulations as described earlier.

5. *In-Vitro* cell line studies of optimised transethosomal formulation

The spontaneously immortalised human keratinocyte line cell line (HaCaT) was provided by the National Centre for Cell Sciences (NCCS), which has its headquarters in Pune, India. In T-75 flasks filled with DMEM (Dulbecco's modified eagle media) and containing 1 percent and 10 percent v/v of antibiotic antimycotic solution and heat-inactivated FBS, respectively, the cultivated cell line was grown before being maintained at 37°C in a 5 percent CO2 atmosphere. Using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test against HACAT cells, the in vitro toxicity of 5-FU, CVD, and FU-CVD TEs was assessed. Further below studies as per ideal protocol.

i. MTT assays
ii. Cell Cycle Analysis
iii. Cellular Apoptosis
iv. Reactive Oxygen Species (ROS) Analysis
v. wound healing
vi. Cell Internalisation study by using FITC, DAPI, MARGE

6. Preparation and optimization of CVD-FU loaded Transethosomal hybrid gel

Optimization of the concentration of gelling agent

Prepare TEs gel, Carbopol 934 and poloxamer 188G ratio (75:25) was added to water under continuous stirring overnight at 1200 RPM using a magnetic stirrer (Remi Motors Ltd., India). The base of Carbopol 934 was then neutralized using triethanolamine (0.05% w/w) for developing gel. The developed CVD-FU-TEs was then added to the gel under vigorous stirring. 0.01% sodium benzoate was finally added as preservative in the preparation.

7. Characterization of CVD-FU loaded Transethosomal hybrid gel

i. Homogeneity

Gel Homogeneity was inspected by visual observation. They were tested for their appearance and presence of any aggregates.

ii. Grittiness

All the formulations were evaluated microscopically for the presence of particles if any no appreciable particulate matter was seen under light microscope. Hence obviously the gel preparation fulfils the requirement of freedom from particular matter and from grittiness as desired for any topical preparation.

iii. Viscosity

The measurement of viscosity of the prepared gel was done with a Brookfield viscometer. The gels were rotated at 20 and 30 rpm using spindle no. 64. At each speed, the corresponding dial reading was noted.

iv. pH evaluation

Gel formulations weighed in 100 mg was made to 50 ml with double distilled water. A glass microelectrode was inserted into the formulation to measure pH.

v. Texture profile analysis of gel

Software regulated texture analyser (Food technology corporation, Virginia, USA) was utilized for the evaluation of texture profile analysis of gel. Fifty ml of the formulated gel was transferred to a 100 ml beaker to avoid entrapment of any forms of air bubbles and ensure smooth surface. The gel in the beaker was then compressed by analytical probe twice up to a depth of 15 mm at 2mm/s rate with a delay interval of 20 seconds in between the termination of first and initiation of second compression. The force-time curve obtained at the end indicates the mechanical parameters of the gel such as hardness, compressibility, cohesiveness, adhesiveness and elasticity. All experiments were carried out in triplicate.

vi. Extrudability

The extrudability was measured by observing weight in grams applied on a collapsible tube containing gel formulation to extrude the gel content in 0.5 cm within 10 seconds.

vii. Spreadability

The gel formulation must have sufficient spreadability characteristics to qualify the status of gel. This is generally expressed as areas covered on the application site such as skin by gel on accounts of its spreadability characteristics. The specific technique used to measure the spreadability is as follows. A glass plate pre-marked with a circle having 2 cm was taken. The gel formulation weighed in 0.5g gram was cautiously placed in that circle and another second glass plate was placed upon the first glass plate. Then a weight of 500 g was put upon the upper glass plate and left as such for 5 minutes.

viii. Drug content

The drug content of the gel formulation was measured by HPLC method by appropriately dissolving 1 gram of gel in 100 ml methanol taken in volumetric flask. The measurement was taken in triplicate and was expressed as percentage of drug content.

ix. Rheological Studies:

The rheological characteristics of CVD-FU-TEs gel were determined. Firstly, the flow behavior of CVD-FU-TEs was demonstrated through a renogram.

x. Texture profile analysis

The texture profile analysis of CVD-FU-TEs gel was based on consistency, firmness, cohesiveness, and work of adhesion.

xi. *In-Vitro* diffusion studies

The In vitro permeation study was performed to determine the release of the drug from the formulation. Such study of Transethosome was performed by using Franz diffusion cell having a capacity of 25ml. Before using dialysis, membrane was soaked in phosphate buffer solution (PBS) of pH 7.4 for overnight. Diffusion cell was filled with PBS pH 7.4 and dialysis membrane was mounted on cell. The temperature was maintained at 37 ± 0.5°C and Maintained speed of magnetically stirrer. Optimized TEs solution, TEs Gel, marketed formulation was placed in the donor chamber. The samples (0.5mL) were withdrawn at suitable time intervals over a period of 4h (0, 1, 2, 3, and 4h) and replaced with the equal amount of fresh PBS (pH 7.4) to maintain

sink condition. The drug concentration on the receptor fluid was determined spectrophotometrically against appropriate blank. The experiment was carried out in triplicate.

xii. *Ex-vivo* drug release study on human cadaver skin

Ref. Same as explained before studies

8. ***In-Vivo* studies**

i. Animals

The Institutional Ethical Committee (SPTM/2022/IAEC/12), a recognized laboratory animal facility in Shirpur, India, gave its approval to the experiment's protocol. Also, animal handling and care followed the standards established by the Indian National Science Academy in New Delhi and the World Health Organization in Geneva, Switzerland (India). A Male Wistar Albino Rats (About 6-to-7-week age and weighing between 120 to 150 g.) chosen from an inbred colony of the random breed was used in the current investigation. Mice were kept in a temperature-controlled environment at 22–25 °C in individual crate systems made in the institute. Food and water were provided on a free-choice basis, and the animals were maintained in a 12-hour light/dark cycles.

ii. Skin Irritation Study

determine skin irritation of CAR Gel, FU Gel, and CAR-FU optimized gel in rats, Draize score test was employed. Group I was treated with the formalin solution (10%) The vicinal untreated area of rats was used as control skin and the edema and erythema scores were estimated. The formulation was applied to the rat skin, and to assess the edema and erythema, visual scoring was carried out after the sample removal.

Animals will be grouped in two groups of six rats each. Animals in group I will receive 0.5 ml nano vesicular systems of anticancer drugs on a surface of 1 cm 2 on skin. Animals in group II will receive 0.5 g of nano vesicular gel systems of anticancer drugs on the surface of 1 cm 2 on the skin. Approximately 24 hours prior to the test, dorsal surface of the animal will be shaved, carefully, preventing any abrasion to the skin. The area for shaving will be such that that animal will not be able to scratch its back. Each animal will be housed individually. After the test animals will be observed for a total period of 14 days. Each animal will be examined for signs of erythema and oedema, at 60 minutes, 24 hours, 48 hours, and 72 hours. The oedema and erythema scores will be recorded on the scale of 0 to 4 from no oedema or erythema (0) to severe erythema or oedema (4) as per OECD.

Score Observation of erythema

Score 0 no erythema

Score 1 slight erythema (light pink colour)

Score 2 moderate erythema (dark pink colour)

Score 3 moderate to severe erythema (dark pink colour)

Score 4 severe erythema (dark red colour)

iii. Skin cancer induction

The protocol of the experiment was approved by the Institutional Ethical Committee (1678/GO/a/12/CPCSEA), and animal care and handling were done according to the guideline,

Accordingly, Wister Albino rats will be used for the test. Animals with age between 6 to 7 weeks and weighing between 140 to 150 g will be used for test. Animals will be housed in the animal house facility of the school and animals will be allowed to have standard pallet diet and a free access to purified water, in an environment of 20 0 C temperature and about 50 to 60% relative humidity. The 12 hours dark – light cycle will be maintained. Approximately 24 hours prior to the test, dorsal surface of the animal will be shaved, carefully, preventing any abrasion to the skin. The area for shaving will be such that that animal will not be able to scratch its back. Each animal will be housed individually. The dorsal side of the animals will be exposed to UV radiations of UV chamber by maintaining around 20 cm distance between the lamp and the cage. Animals will be divided into five groups each composing six animals.

- Animals in the first group will serve as UV control. They will receive the UV radiation but no
- any treatment. Animals in the second group will be treated as standard group. Animals in this group will be treated with standard 5 Fluorouracil cream available in the market.
- Animals in third group will be treated with Transethosomal gel of 5 Fluorouracil after getting UV irradiation.
- Animals in group 4 will be treated with Transethosomal gel of Carvedilol after getting UV irradiation.
- Animals in group 5 will be treated with Transethosomal gel of combination of 5 Fluorouracil and carvedilol after getting UV irradiation.

The animals will be irradiated with UV radiation on daily basis for a period of 10 days. This exposure is intended for initiation of tumours in animals. The last exposure of UV radiation (Day 10) will be followed with one week break and again they will be exposed to UVB radiation with an average thrice a week to induce tumour promotion. This time the animals in group 2, to 5 will be treated with cream or gel as described above, about 15 minutes before of each exposure. During this period, they will be observed weekly to find out any possible tumors. The tumours observed in animals will be recorded until their size get stabilized. The incidence of carcinoma will be confirmed by histological examination conducted

9. Stability studies

Stability studies as per the ICH guidelines Accelerated stability studies at different temperature and humidity levels for changes in morphology and drug content. Release study of aged products.

Results

Preformulation Study

Preformulation studies have a significant part to play in anticipating formulation problems and identifying logical path in development process where the Preformulation scientist characterizes the physical, chemical and mechanical properties of a new drug substance, in order to develop stable, safe and effective dosage form. The Preformulation investigations confirm that there are no significant barriers to the compound's development as a marketed drug.

The formulation was using this information to develop dosage forms. Preformulation studies were done to evaluate the purity of drug by physical/morphological examination, melting point, partition coefficient, IR and λmax determination.

Table 3 (a) Preformulation Study

Sr. no.	Evaluation Parameter	Carvedilol	5-Flurouracil
1	**Description**	White Crystalline Powder BCS Class: II	White Crystalline Powder BCS class III
2	**Melting Point**	114-115 ^{0} C	282-283 °C
3	**Partition Coefficient (log P)**	3.05	- 0.89
4	**LOD**	0.201±0.003	0.204±0.003
5	**Wavelength (nm)**	In Methanol 248nm	In water 266nm
6	**Calibration curve Equation**	Y = 0.0996x-0.0065 R^2 = 0.997	Y = 0.059x-0.004 R^2 = 0.998

Table 2 (b) solubility of API

Solvents	5-Flurouracil (mg/ml)	Carvedilol (mg/ml)
Water	12.2	0.5
95% Ethanol	5.5	1.5
Chloroform	< 0.1	1.5

A simple, quick, and cost-effective RP-HPLC technique for determining CVD and 5-FU has been developed. The suggested technique was validated in accordance with the ICH guidelines and was shown to be very accurate, precise, and robust for estimating CVD and 5-FU-based formulations. Forced degradation experiments revealed that neither drug's breakdown products interfered with the chromatograms of CVD and 5-FU. This approach effectively separates both substances. As a result, the method development is a stability indicator. The mobile phase is both simple to prepare and cost effective. This highlights the development of a systematic RP-HPLC technique for simultaneous quantification of CVD and 5-FU in dual drug-loaded transethosomes. As a result, it is possible to infer that the analytical approach has been validated and that it may be used for both stability and routine analysis.

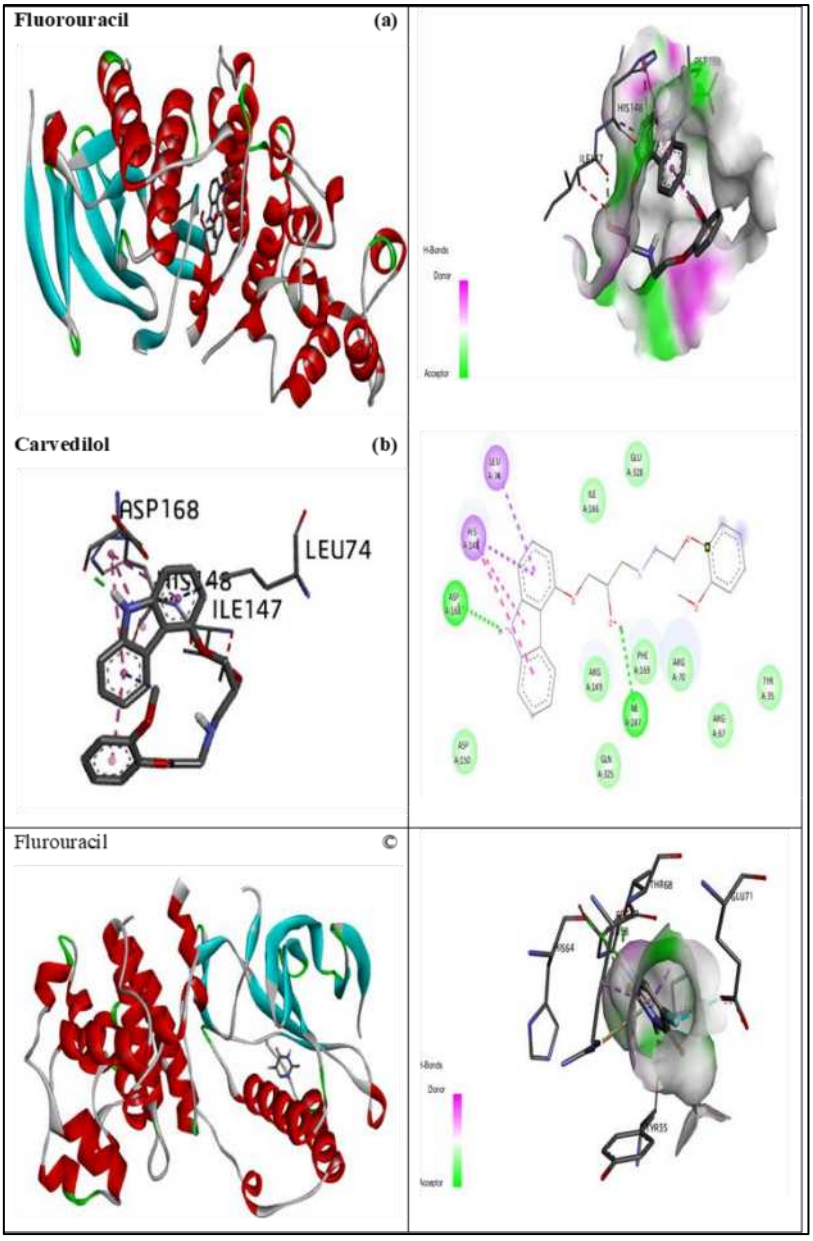

Figure 2 (a) Molecular docking of protein Mitogen-activated protein kinase 14 (2GTM) Binding Domain Complexed with Carvedilol shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction (b)Molecular docking of Protein Mitogen-activated protein kinase 14 (2GTM) Binding Domain Complexed with 5- Fluorouracil shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction

Fourier transform infrared spectroscopy was used to identify the drug and the infrared absorption peaks of CVD, FU, phospholipids to confirm the compatibility between apigenin as a drug with excipients. The spectral scan was carried out in the frequency range from 4000 to 400 cm−1 using a Perkin Elmer FTIR. Data obtained from FTIR Spectrophotometric study clearly indicates in significant changes in spectra obtained from physical mixture of drug and excipients. However no additional peaks other than peaks of individual components were observed. Thus indicates compatibility of Apigenin with selected excipients. The Next step was

the preparation, optimization and characterization of CVD-FU-TEs were successfully formulated by modified Rotary evaporation sonication technique. Using Soya phosphotidylecholine, tween80 and anticancer drug and Different surfactants in different molar ratios were used for the formulation of TEs.

Optimization of CVD-FU-TEs

Fluorouracil and Carvedilol loaded transethosomes was optimised using the surface response BBD model. The optimized formulation has good entrapment efficiency, nanosized vesicle, and flux. Selecting an appropriate design is crucial for characterising and optimising a pharmaceutical preparation. A quadratic surface model is frequently the outcome of the variable demonstration. Selecting an appropriate design is crucial for characterising and optimising a pharmaceutical preparation. A quadratic surface model is frequently the outcome of the variable demonstration. Therefore, for a kind of interpretation, the box-benken design could be an excellent tool. The effect of three independent variables (X1 Lipoid S100, X2 Tween 80 and X3 Ethanol) was ascertained on dependent variables (Y1 Vesicle Size (nm), Y2 Zeta Potential (mV), Y3 % Entrapment efficiency of 5-FU, Y4 %Entrapment efficiency of CVD, Y4 % Skin Permeation of 5-FU, Y4 % Skin Permeation of CVD) and the results were determined statistically using ANOVA. In correspondence with all four responses, the model is found to be fit to use for suggested models. The study showed significantly more ($p < 0.05$) efficient release and permeation of Fluorouracil and Carvedilol loaded transethosomes than conventional form of Fluorouracil and Carvedilol.

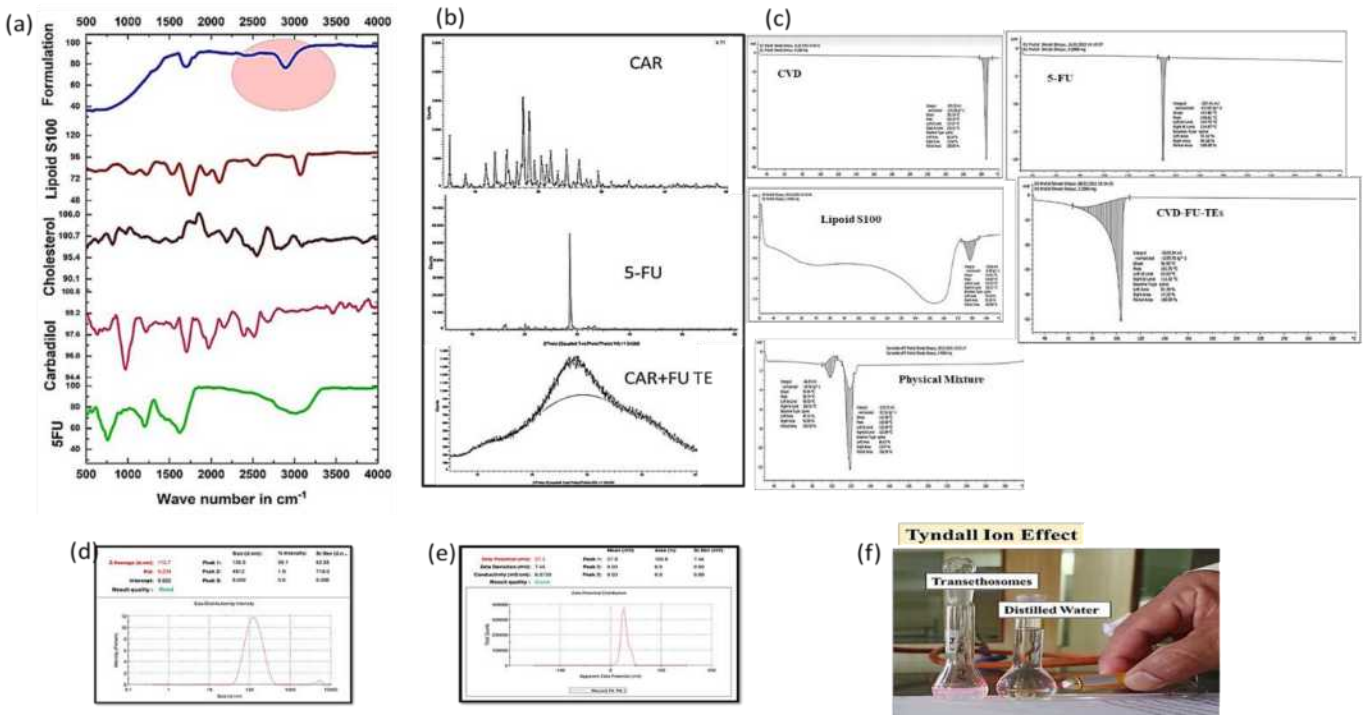

Figure 3 (a) FTIR Spectra of CVD, 5-FU, Lipoid S100, Cholesterol, Optimised Transethosomal formulation (b) XRD spectra of 5-FU, CVD and FU-CVD-TEs (c) DSC of CVD, 5-FU, Lipoid S100, Optimised Transethosomal formulation (d) Particle size of CVD-FU-TEs formulation (e) Zeta potential of Optimised formulation (f) Tyndall Ion effect of TEs Formulation

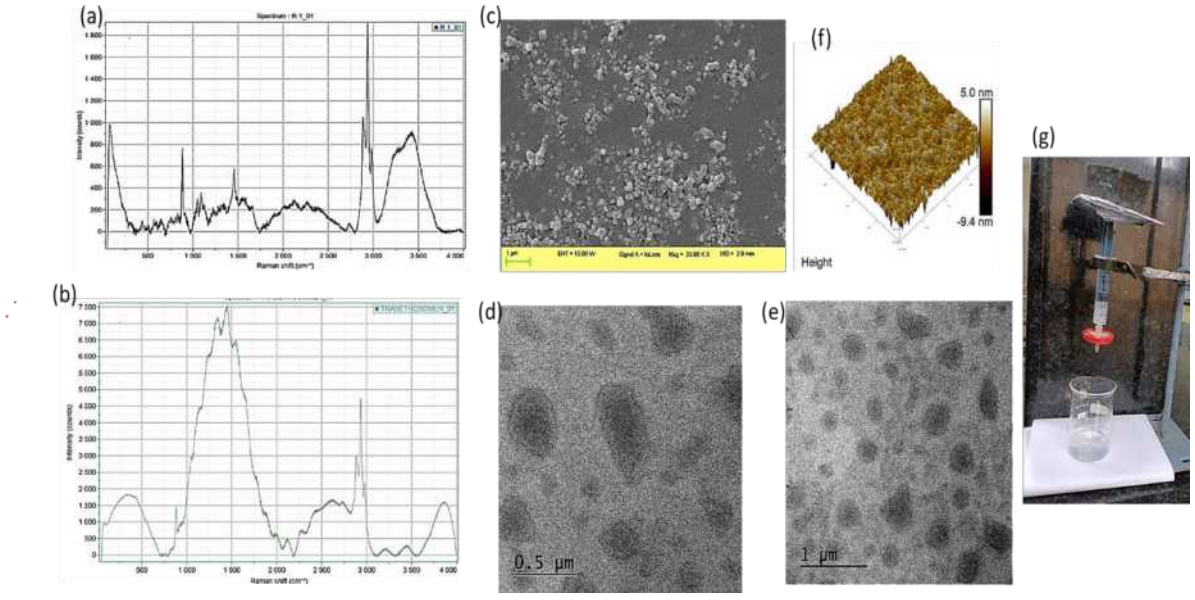

Figure 4 ***(a-b) Raman spectra of Placebo TEs and CVD-FU-TEs Optimized formulation (c) SEM morphology of optimised formulation (d) TEM morphology of optimised formulation 0.5µm and 1µm (e) AFM of CVD-FU-TEs formulation (f) Deformity Index of CVD-FU-TEs formulation (membrane filter 0.22µm)***

After scanning every component in the formulation as well as its physical combination, it was discovered that there was little interaction between the various samples and that the peak of the optimized formulation overlapped with the drug, indicating the existence of the drug in the TEs formulation figure 4 (a-b).

The surface morphology was viewed via SEM imaging which depicted spherically shaped with smooth surface Transethosomes was represented in Figure 4(c).

TEM analysis image of optimized transethosomes formulation revealed that the loaded vesicles are well-identified sealed structure with uniform size distribution and spherical in shapes shown in figure 4 (d).

AFM was used to observe the surface topography and morphology of the TEs. The parameters, such as average roughness (Ra) and root mean square roughness (Rq), were obtained from AFM software analysis. are relatively flat and smooth, it seems that the surface is mainly composed of mountain-like protrusions, and the surface has become rather rough, with the Ra and Rq increased to 3.38 and 4.52 nm respectively. Increased the roughness of the composite surface shown in figure 4 (e).

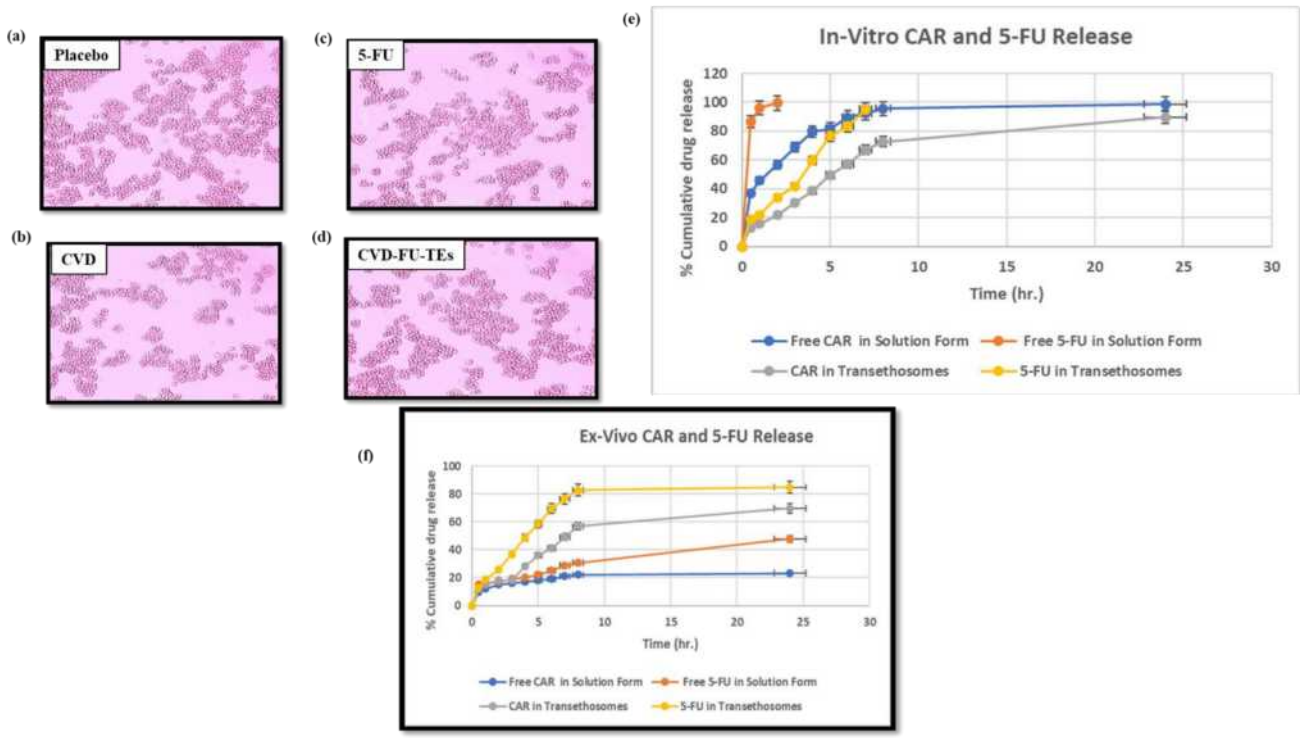

Figure 5 ***(a-d) Histopathology and platelet aggregation studies of Placebo TEs, CVD, 5-FU CVD-FU-TEs Optimized formulation (e) In-Vitro release of CVD-FU-TEs formulation (f) Ex-Vivo drug release of CVD-FU-TEs formulation***

The experimental interpretation discussed above makes one thing very clear: polysorbate 80 is hemocompatible, but greater quantities of polysorbate 80 can result in hemolysis. Erythrocyte solutions treated with 1% Triton X-100 are shown in Figure 5(a) as the positive control, untreated erythrocyte solutions as the negative control, and the treatments are FU-CVD, placebo, and FU-CVD loaded transethosomes. improved formulation Figure 5(b)- shows that FU-CVD-TEs demonstrated >0.5% hemolysis and were deemed safe for RBC membrane integrity 5(c). Due to the transethosomes effective FU-CVD encapsulation within the nanoparticles, which shields red blood cells from FU-CVD-induced haemolysis, there was reduced haemolysis detected in these transethosomes.

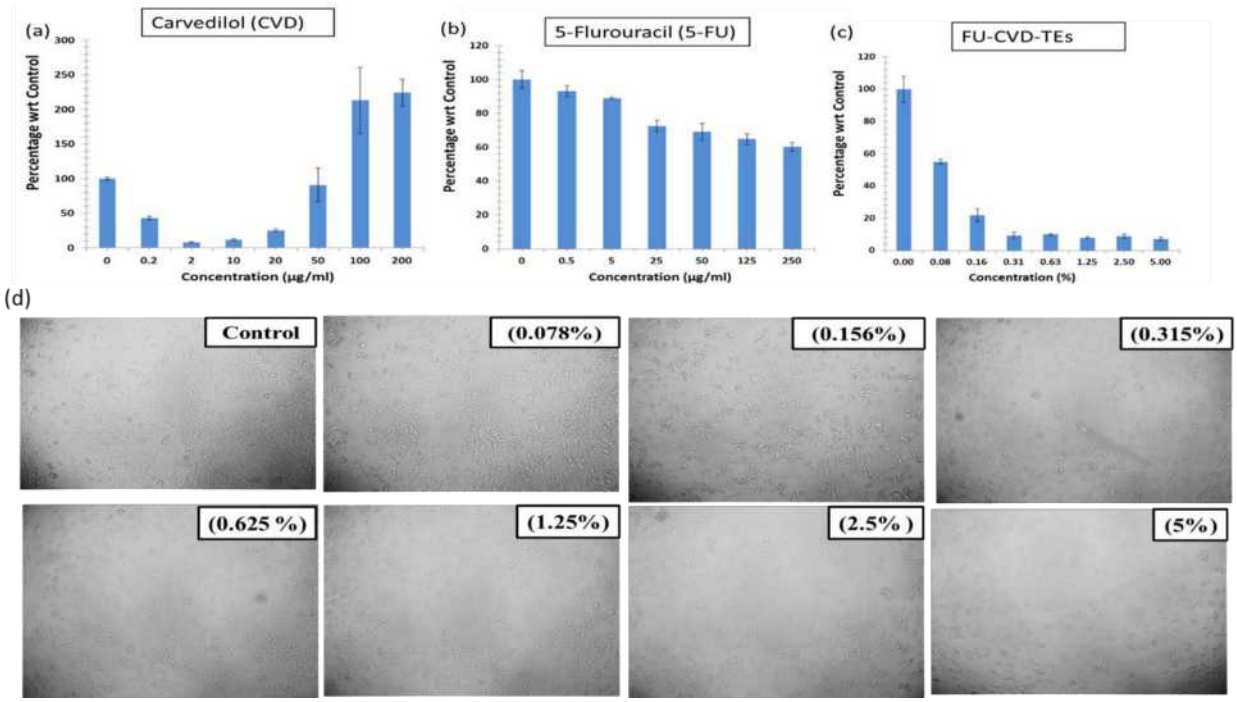

Figure 6 Effects of tailored transethosomes (TEs) on the HaCaT Cell line in vitro cytotoxicity. (a-c) FU-CVD concentration-dependent transethosomes cell viability of HaCaT cells (%) and (d) Effect of transethosomes drug concentration-dependent cell under optical microscopy

It is crucial to assess the cell cytotoxicity of various formulations at various concentration levels prior to moving on to the in vivo anti-cancer investigation. The immortalised human keratinocytes cell line (HACAT) revealed concentration-dependent cell cytotoxicity. The results showed that the IC50 for 5-FU, CVD, and FU-CVD-TEs, respectively, was 220 g/ml, 0.1555 g/ml, and 0.0668%. (Fig. 6a-d). As shown, the IC50 value of TEs is more than 5-FU because it is a powerful anti-cancer drug that promotes cell death by activity, thymidylate synthase act cells, but it is lower than the non-TES preparation.

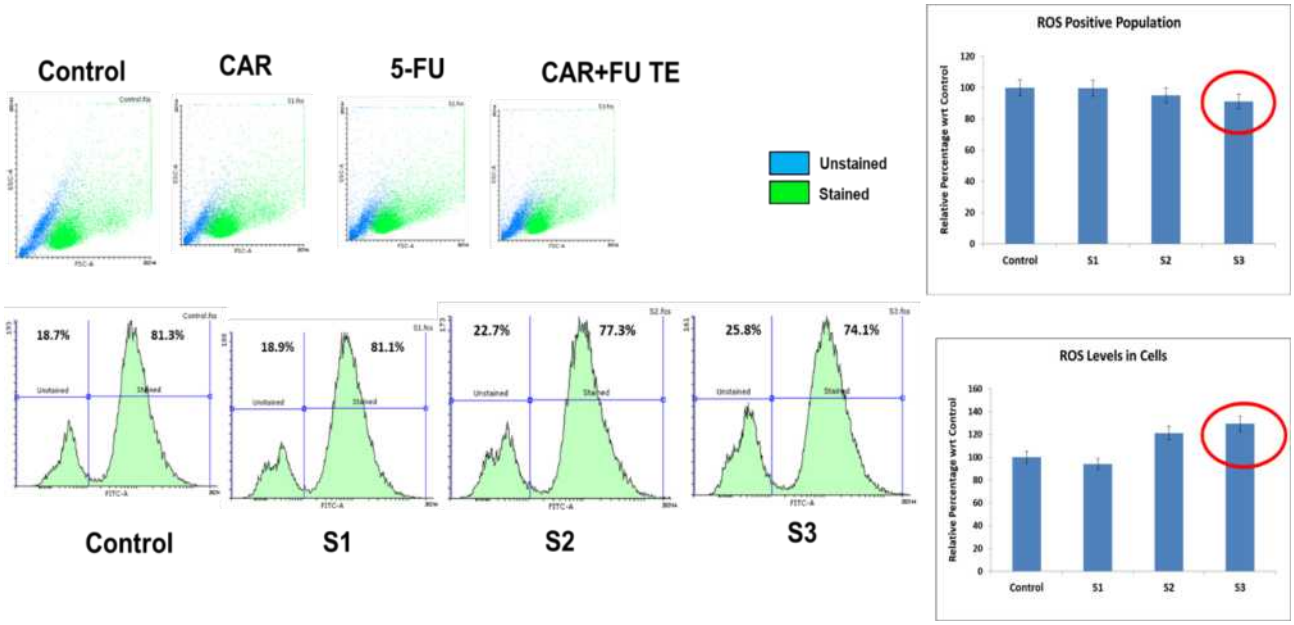

Figure 7 ROS Estimation with Flow Cytometry

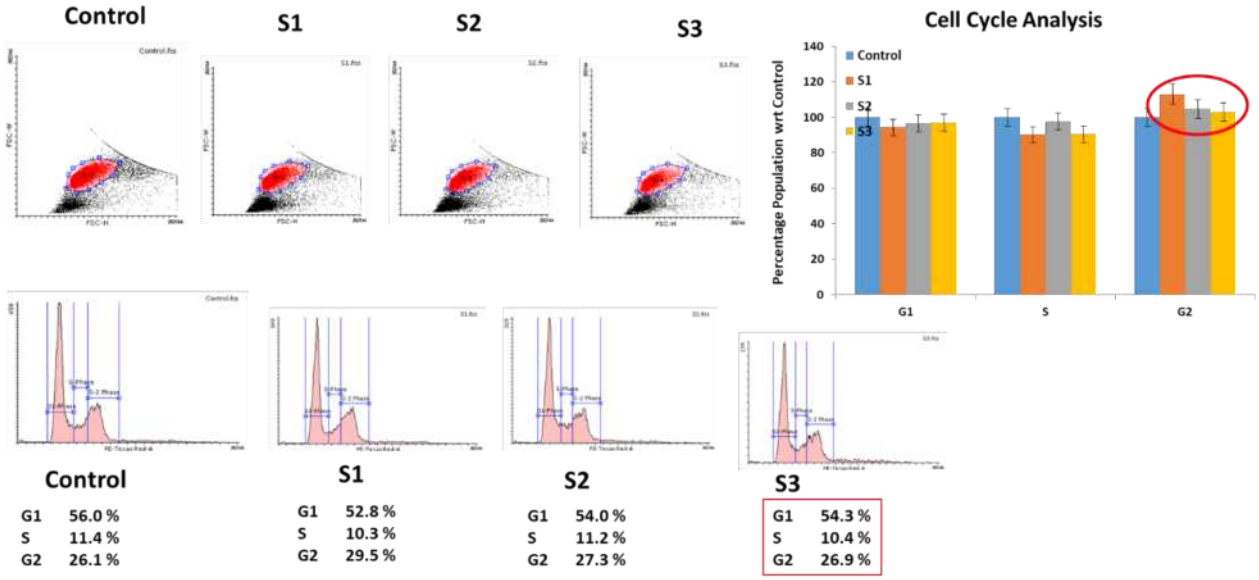

Figure 8 Cell Cycle Analysis with Flow Cytometry

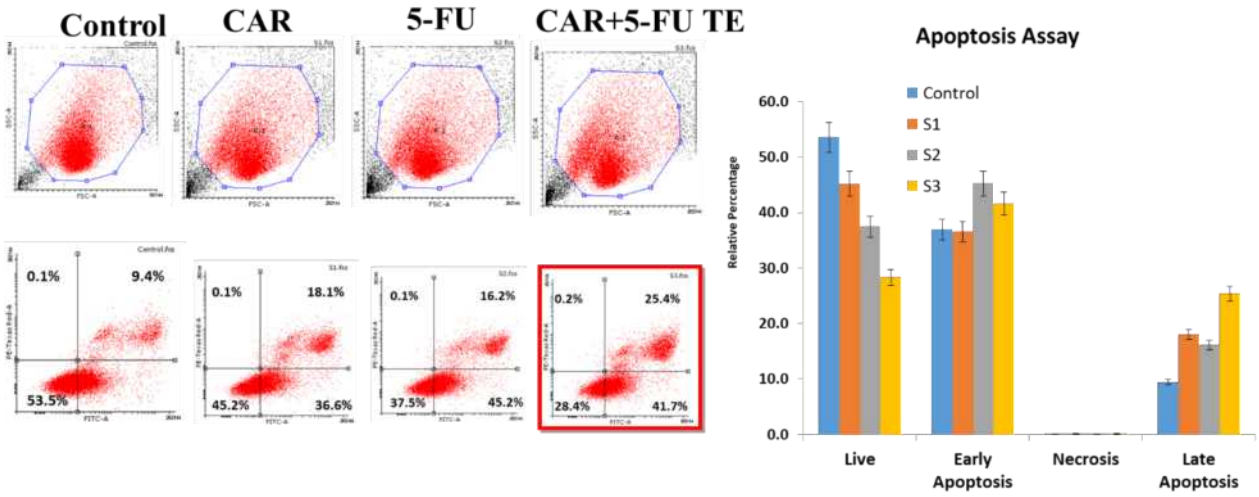

Figure 9 Cellular Apoptosis with Flow Cytometry

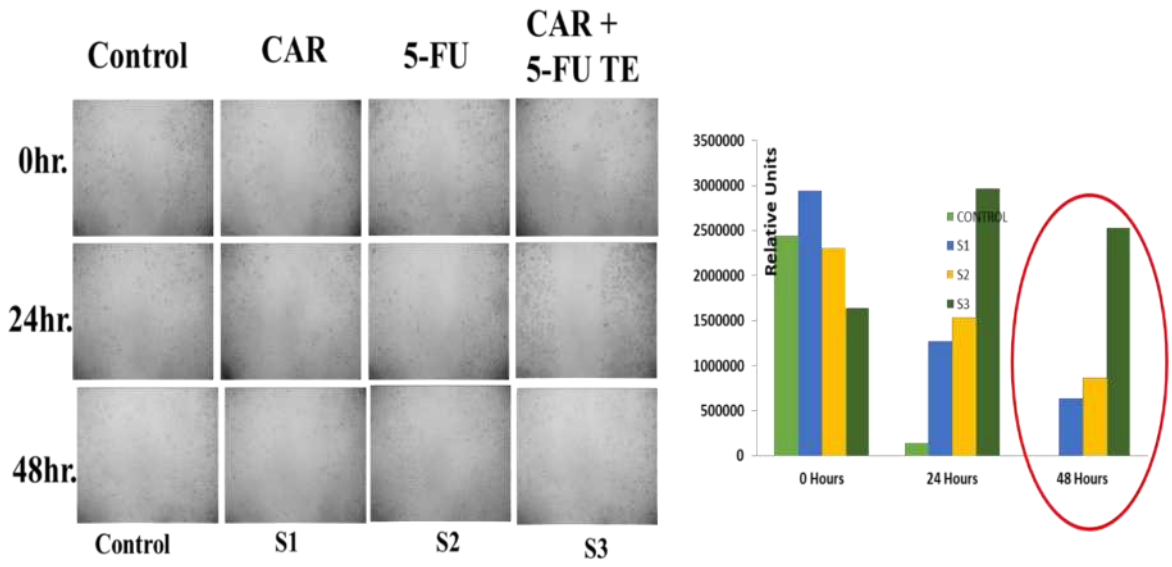

Figure 10 In Vitro Scratch Assay

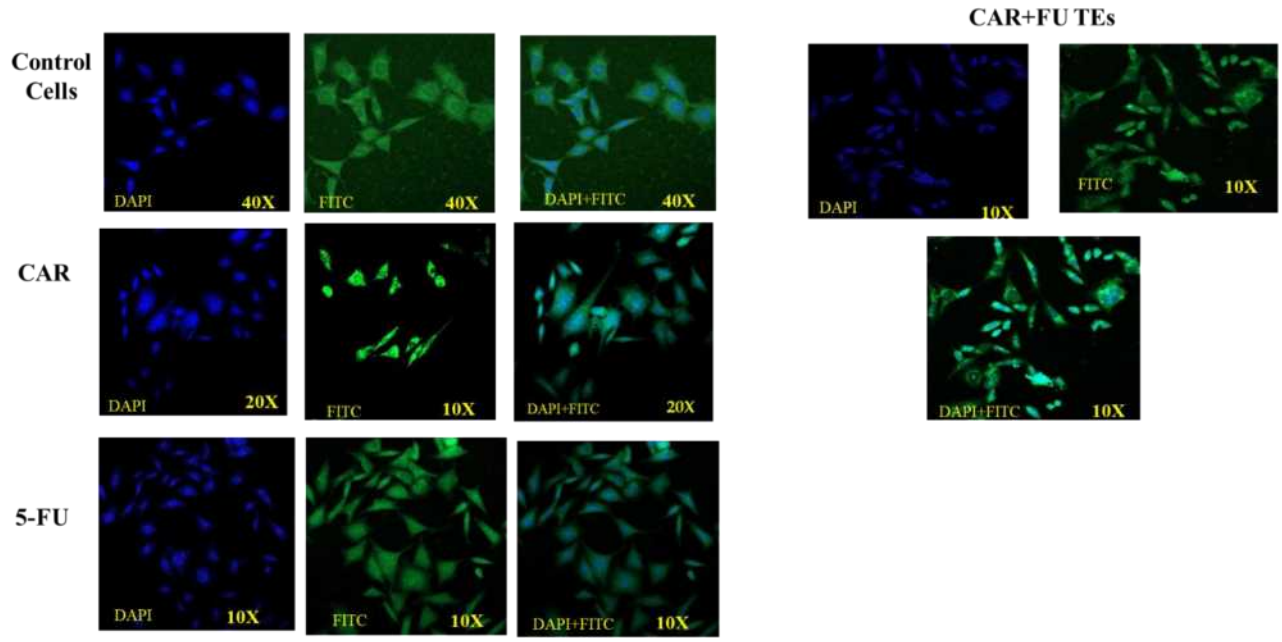

Figure 11 Cellular Uptake of Compounds in HaCaT Cell Lines

Figure 11. was shown that the cells of the control group showed normal nuclei, however, cells treated with CVD-FU-TEs showed more shrinkage, apoptosis, and fragmented nuclear morphology than CAR and FU. A comparative result of CVD-FU-TEs with CVD and FU is due to slow release, higher internalization, and greater accumulation of TEs into the cells. From the results, it was evaluated that CVD-FU-TEs could be a potential candidate for amelioration the skin carcinoma.

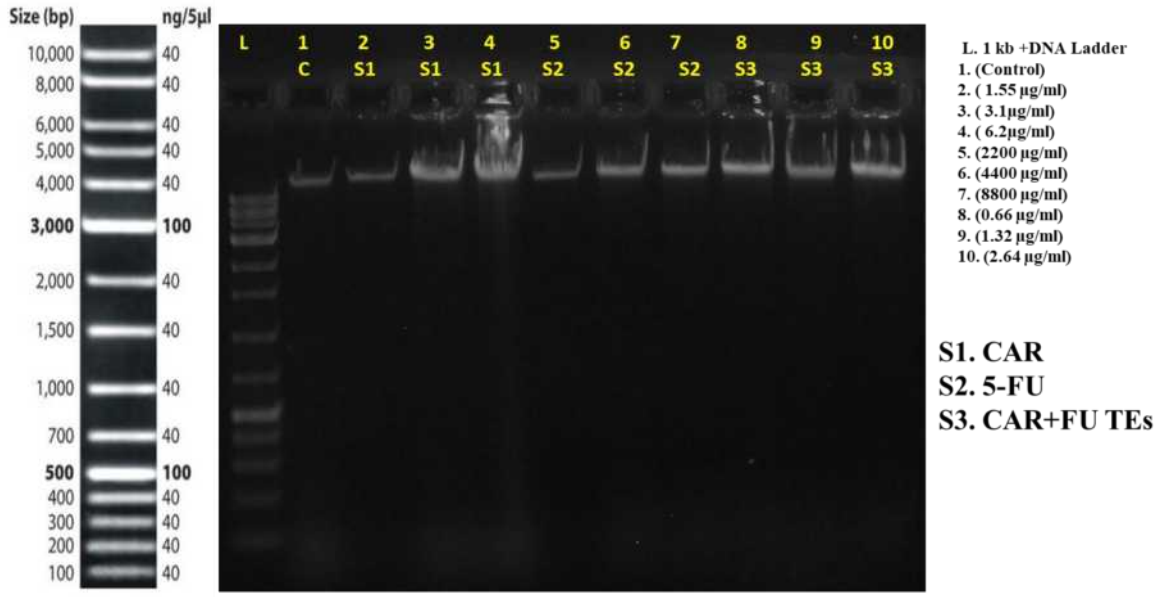

Figure 12 ***DNA Fragmentation Assay***

DNA Fragmentation Assay was indicated (Figure12) that CAR and 5-FU caused DNA fragmentation and cancer cell death, in combination also increased the cleavage of caspases

9, 8, and 3, which break DNA in cancer cells. Furthermore, treatment of HACAT cells with CAR+FU TEs increased the amount of DNA fragmentation compared to untreated cells. Based on the experimental findings, encapsulating CAR+FU into TES can markedly enhance the anti-tumor effect of CAR+FU in skin cancer cell lines. Consequently, our results show a novel approach for enhancing chemotherapeutic drug efficacy and overcoming the systemic side effects of this drug.

Optimization of CVD-FU-TEs Hybrid gel

Since it has strong cohesive properties and is simple to wash off, suitable quantity of Carbopol 934 and poloxamer 188 G was used to make CVD-FU-TEs gel. Based on the pH, physical inspection, and homogeneity optimised gel was assessed. The generated CVD-FU-TEs gel was then subjected to rheological studies, texture profile analysis, and content uniformity, extrudability, and spreadability analyses.

Table 4 Evaluation of CVD-FU-TEs optimised hybrid gel evaluations

Parameters	Result
Clarity and Homogeneity	Clear and homogeneous appearance
Washability	Easily washable without leaving any residue on the surface of the skin
Greasiness	Non-greasy
Grittiness	Free from grittiness
Ease of application	Easily/smoothly applied
pH	6.72 to 7.10
Viscosity	10632 ± 1.78 cps
Extrudability	Excellent
Spreadability	Excellent with 90% spreadability

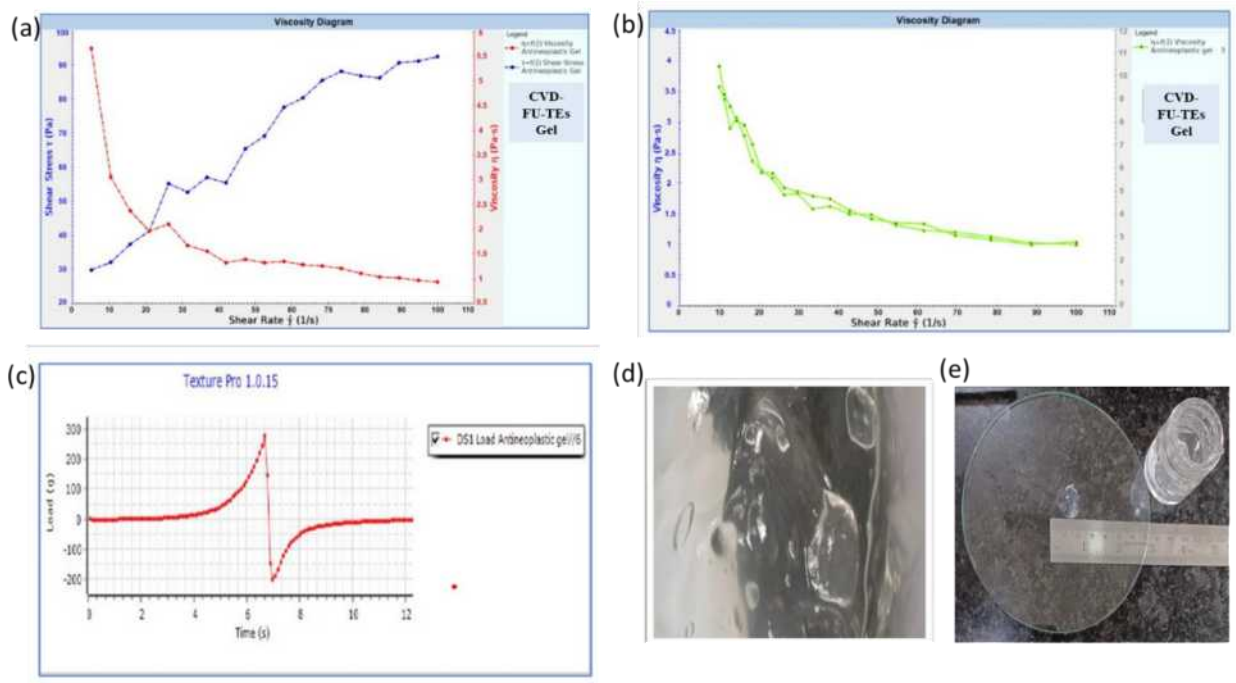

Figure 13 evaluation of CVD-FU-TEs optimised hybrid gel evaluations (a-b) Rheology (c) Texture profile of gel (d) physical observation of gel

In short, if the gel formulation has reached a specific phase and viscosity and is held at room temperature, it may be transported readily provided that it is not subjected to any shear changes that could modify its viscosity, stability and structure. A CVD-FU-TEs gel better adhesion results in longer skin contact time, which might mean using the gel less often.

In vivo studies

Table 5 In-Vivo studies: Skin Irritation Study

Groups	Erythema		
	24h	48h	72h
Group I (Control)	0	0	0
Group II Standard 5-FU	0	0	0
Group III CAR TEs Gel	0	0	0
Group IV 5-FU TEs Gel	0	0	0
Group V CAR+ FU TEs Gel	0	0	0

Figure 14 In-Vivo studies: Skin Irritation Study

The skin irritation potential of CAR TEs gel, 5-FU TEs Gel and combination of CAR+FU TEs Gel was assessed by carefully examination of skin erythema at the application site of skin. The formulations such as CAR TEs gel, 5-FU TEs Gel and combination of CAR+FU TEs Gel had scored zero during the examination shown in Figure 13. This displays the suitability of formulations for topical delivery of medicament without inducing any skin irritation at the application site.

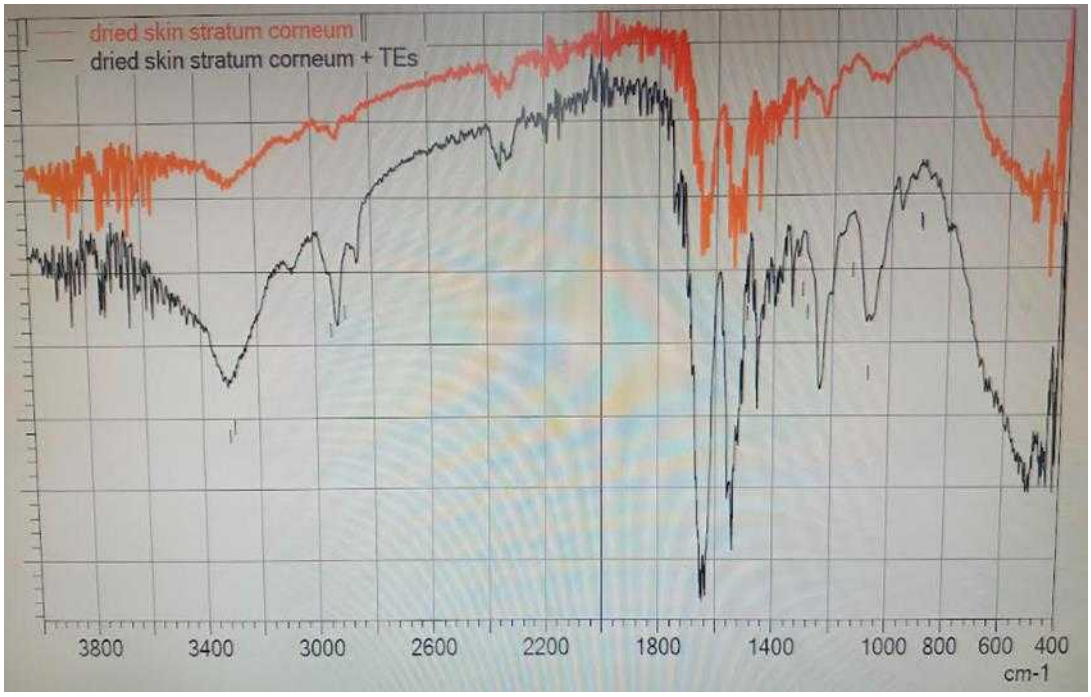

Figure 15 FT-IR spectra of rat skin after 8 h. (A) Untreated dried skin (B) TEs formulation

Alteration in the fluidity of stratum corneum was observed by focusing on the region near to 2850 cm^{-1} and 2920 cm^{-1} as shown in Fig. 3. In the case of TEs gel formulation, it may be concluded that the peaks obtained near at 2850 cm^{-1} and 2920 cm^{-1} were due to the C–H symmetric stretching and C-H asymmetric stretching absorbance, with slight alterations, as shown in figure.

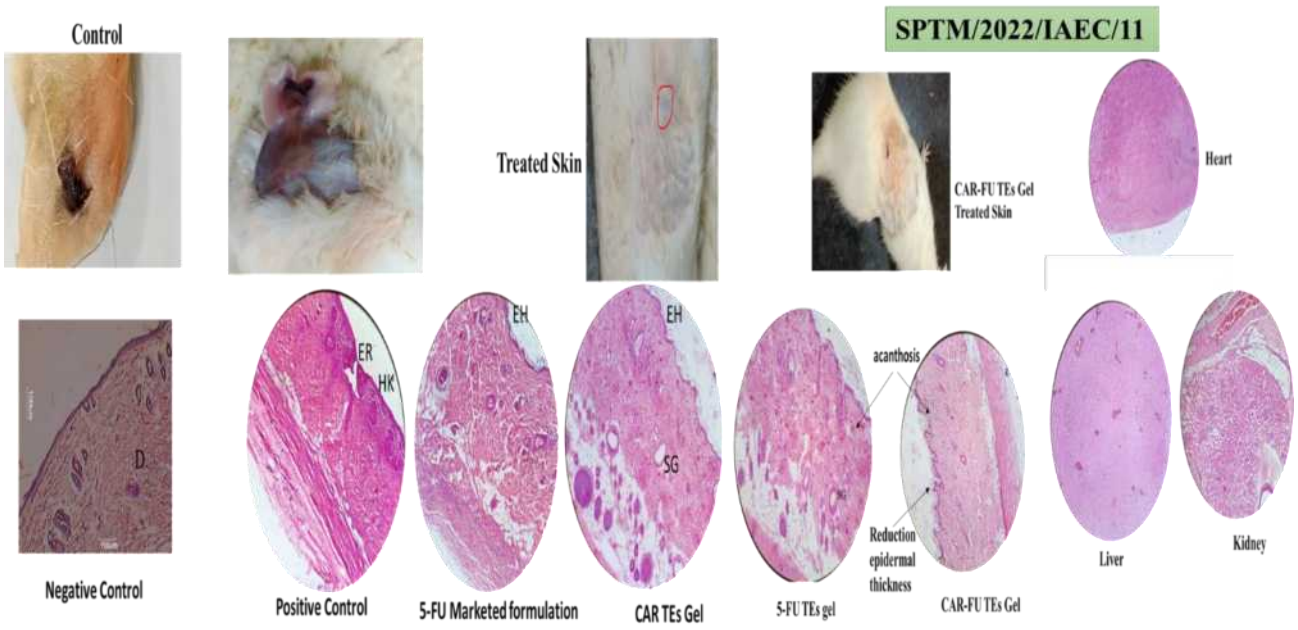

Figure 16 Study on skin UV irradiation (as models of skin cancer) of TEs gel systems of anticancer drugs on rats

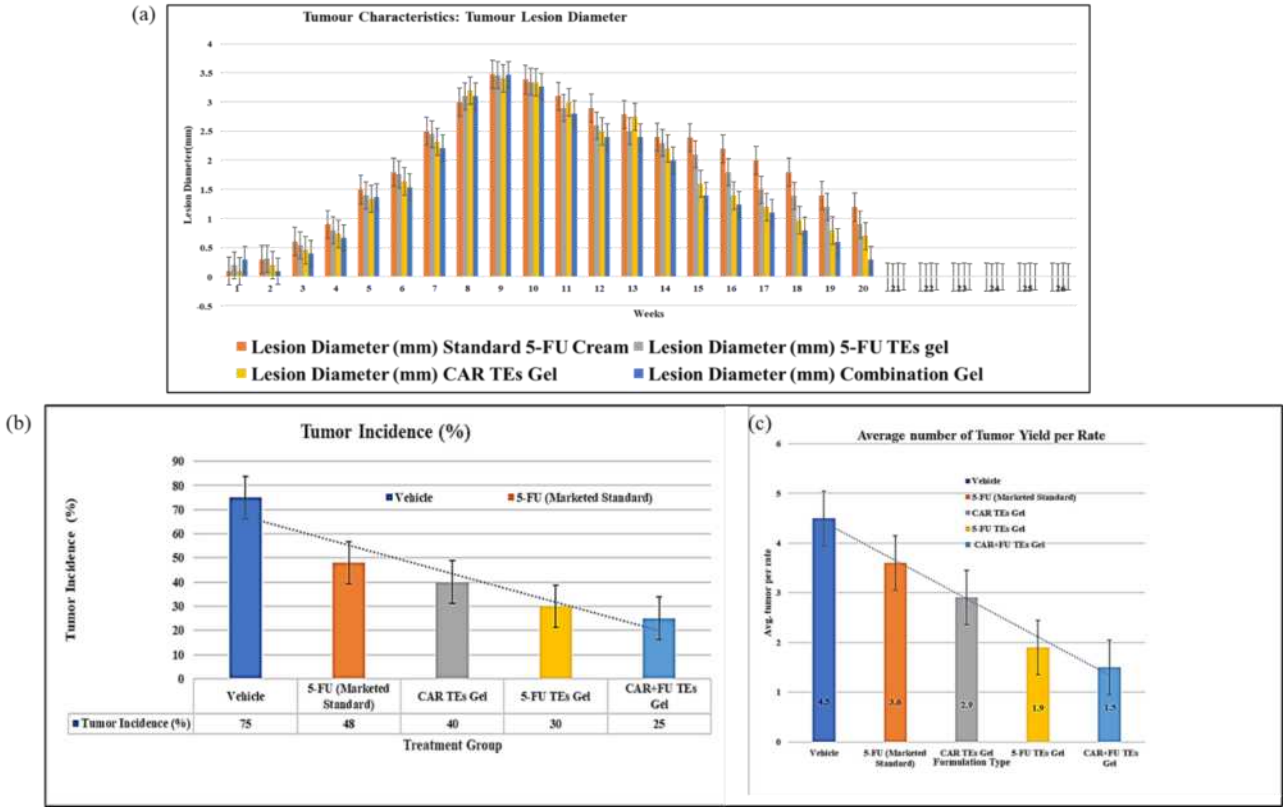

Figure 17 (a) ***Tumour Characteristics: Tumour Lesion Diameter (b) % Tumor incidence (c) Average Numbers of tumor yield per rats***

In histological investigation, the skin of the gel-treated groups had normal-appearing epidermis and extensive lymphoplasmacytic infiltrates in the subcutaneous region. These show the treated groups have returned to their natural morphology. According to OECD (402) requirements, a safety investigation of the topical gel formulation was conducted on rats utilizing the acute dermal toxicity technique. At 2000 mg/kg body weight, no treatment-related mortality, unusual clinical symptoms, or notable changes in body weight were seen. None of the experimental animals had any obvious pathological findings.

A histopathological analysis of mouse skin revealed considerable epidermal inflammation, penetration of inflammatory epidermal cells into the dermis, and the development of keratin pearls on skin that had just been exposed to UV radiation. However, there were no keratin pearls or epidermal cell infiltration on the skin of mice treated with the OFEF gel formulation. Studies on skin sensitivity after using the OFEF gel formulation indicated that the mice received no points for this examination. This guarantees that the created formulation of the OFEF gel is painless and secure for topical use.

Stability Studies

The developed in lab CVD-FU-TEs Hybrid gel formulations were stable on storage at 4±2 ^{0}C and 25±2 ^{0}C/60±5% RH for six months. The calculated shelf life of CVD-FU-TEs Hybrid gel at 4±2 ^{0}C and 25±2 ^{0}C/60±5% RH were 917 days and 460 days respectively.

Statistical Analysis:

All data are presented as mean standard deviation (SD). The two-way ANOVA was used to determine the statistical significance of variations between more than two groups. Values between $P < 0.05$ and $P < 0.001$ were found to be significantly and more precisely significant, respectively.

Discussion:

The work undertaken, presents the findings of a scientific study on 5-Fluorouracil (5-FU) utilising UV spectrophotometry, RP-HPLC, and a range of analytical techniques. The method of UV spectrophotometry exhibited remarkable linearity and sensitivity in quantitative analysis, whereas the RP-HPLC method showcased exceptional precision, accuracy, and robustness in estimation. The stability and drug loading efficiency of the CVD-FU-TEs were enhanced through optimisation. Characterization techniques, including transmission electron microscopy (TEM), atomic force microscopy (AFM), and confocal microscopy, were

employed to gain insights into the morphology and structure of the emulsion. The research presents significant insights into the analysis, optimisation, and characterization of CVD-FU loaded in vesicle, with the aim of exploring its potential applications in drug delivery systems. In this manuscript, we explore into the rheological properties of Transethosomes consist of CVD-FU-TEs. Additionally, we explore the rheological properties of an CVD-FU-TEs Hybrid gel. The analysis of frequency sweep revealed that the gel composed of CVD-FU-TEs demonstrated a greater storage condition and displayed characteristics similar to a solid, suggesting its stability. The storage condition of the formulations was positively correlated with the concentration of CVD-FU-TEs and the presence of Carbopol 940 and Poloxamer 188G in the gel, thereby enhancing the stability of the formulations. The Transethsomes and gel exhibited shear-thinning behaviour, and the apparent viscosities were enhanced by the inclusion of the poloxamer 188G. The presence of relevant functional groups in the formulations was confirmed through Fourier transform infrared spectroscopy (FT-IR). The results obtained from the differential scanning calorimetry (DSC) analysis revealed the absence of any endothermic peaks, indicating the successful encapsulation of CVD and 5-FU. The X-ray diffraction (XRD) analysis indicated that the crystalline structure of the Transethsomes and gel remained unaffected by the Polyvinyl alcohol encapsulation. In vitro drug release studies revealed sustained release patterns, with the release mechanism identified as Fickian diffusion. The cytotoxicity of the CVD-FU-TEs formulation against the HACAT cell line was determined using the Sulforhodamine B (SRB) assay. The results showed that this formulation had the lowest IC50 value, indicating its higher cytotoxicity. The analysis of the cell cycle revealed that all treatments resulted in the arrest of the G1 phase. Among them, the treatment with Anti-CVD-FU-TEs exhibited the most pronounced effect. The apoptotic and necrotic activities of the formulations were further confirmed through flow cytometry analysis. Among them, CVD-FU-TEs exhibited the most significant apoptotic effect. The measurement of reactive oxygen species (ROS) revealed that the CVD-FU-TEs formulation resulted in lower ROS levels in comparison to CVD and 5-FU. This suggests that the CVD-FU-TEs formulation has a lesser effect on ROS production. Among the formulations, CVD-FU-TEs exhibited the most significant effectiveness. The results underscore the remarkable anticancer effects of the CVD-FU-TEs formulation and its potential as a promising therapeutic option. We further discuss several aspects of a gel formulation, including its pH, rheological properties, spreadability, texture profile, and *In-Vivo* studies. The presence of DNA ladders in cells treated with CVD, 5-FU, CVD-FU-TEs was confirmed through the DNA fragmentation assay, indicating the induction of apoptosis. The wound healing assay showcased the capacity of Anti-EGFR-5-FU-

CS-PE to impede the migration of cancer cells. The characterization of the gel formulation revealed favourable pH, viscosity, spreadability, and texture properties. Skin irritation studies conducted on rats demonstrated that the gel exhibited a non-irritating nature, thereby establishing its safety for topical application. In vivo studies conducted on rats with experimentally induced skin lesions revealed that the gel exhibited significant efficacy in inhibiting tumour growth, The results indicate that the CVD-FU-TEs gel formulation holds promise as a viable cancer treatment option. Further research is warranted to explore the molecular mechanisms and clinical efficacy of the subject matter.

Impact of the research in the advancement of knowledge or benefit to mankind

Nanomedicine for skin cancer has already become a current scientific practice, and it has demonstrated a major evolution in the treatment of skin tumors, including aggressive and invasive tumors. The problem of resistance to chemotherapeutic agents is perhaps one of the greatest challenges in clinical medicine. As a result, the Nano vesicular delivery system has benefits such as specific passive targeting of tumour tissues, increased effectiveness and therapeutic index of medicinal molecules, increased stability by encapsulation, and reduced side effects of encapsulated drugs. It also exhibits a site avoidance effect, enhances drug molecule pharmacokinetic characteristics (lower elimination, increased circulation life durations), provides flexibility in coupling with site specific ligands to accomplish active targeting, and aids in reducing harmful drug exposure to healthy tissue. The developed vesicular system was in liquid form, thus having less viscosity which could not be applied topically. Therefore, it is incorporated into selected hybrid hydrogel such as Carbopol 934 and poloxamer 188G. Another approach, hybrid gel has been shown to be the most feasible, efficient, and effective method of distribution. It also leads to better therapeutic release. formulation that takes more directed (site-specific) action as opposed to randomly design showed continuous release while avoiding cytotoxic drugs negative effects. Reducing frequent dosage that ultimately resulted in cost savings developed a new field of study in relation to medication development, offers a better patient compliance option. An innovative drug delivery method against skin cancer that gave a promising price economic prospects for cancer medicine delivery systems.

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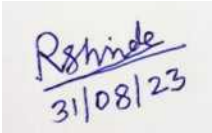

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