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FORMULATION OF NANOSTRUCTURED BASED DELIVERY SYSTEM OF FISET IN FOR SKIN CANCER

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ABSTRACT: The research work is focused on the formulation of dermal drug delivery system of Fisetin. The formulation based on the principal to causes deeper skin penetration and minimal systemic delivery for the management of skin cancer by enhancing the dermal bioavailability of formulation with reducing dosage requirement in sustained delivery mode by direct targeting of formulations to the skin. Two formulations *viz* transethosome and ethsosme were formulated to attain the efficient dermal delivery of fisetin. The surface morphology was studied by using Zeta sizer procedures. The formulation had shown adequate strength, consistency, cohesiveness, and list of thickness as 330.50 g, 314.69 g, 2.34.50 g, and 112.71 g separately. The streamlined fisetin transethosome gel definition detailing had 90% spreadability and a pH of 6.5 ± 0.2. These properties guarantee uniform spreading of OFTF-gel at the application site without causing skin irritancy. OFTF-gel had shown great substance consistency with 99.5 ±1.5 % of medication recuperation from the gel by HPLC assessment. The skin treated with transethosomes gel had shown altogether higher in the epidermis and dermis when contrasted with the ordinary gel.

INTRODUCTION: The non-melanoma skin cancers comprise the cancers affecting the skin apart from melanoma, counting basal cell carcinoma and squamous cells carcinoma AS the most commonly diagnosed and malignant one [1]. Among which squamous cells, carcinoma is regarded as the most malignant because of its invasive nature, which can lead to metastasis and end up in the death of the patient. Solar ultraviolet radiation (UV) is considered the most prominent contributing factor of the aforementioned cutaneous malignancies. Alterations in lifestyle, dietary habits, and environmental conditions also can arise AS risk factors of skin malignancies. The skin cancer diagnosis has increased alarmingly in many countries [2].

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The research focuses on the formulation of a dermal drug delivery system of fiset with deeper skin penetration capability and minimal systemic delivery for the management of skin cancer. We tried to enhance the dermal bioavailability of formulation by reducing dosage requirement in sustained delivery mode by direct targeting of formulations to the skin. Two formulations, *viz*

transethosome and ethsosme were formulated to attain the efficient dermal delivery of fisetin.

MATERIALS AND METHODS:

Materials: Fisetin was obtained as a gift sample of Ranbaxy Laboratories Limited, New Delhi. All other materials and reagents were of the analytical grade of purity.

Methods:

Preparation of Vesicular Formulations: It occurs in two steps. First, a thin film is prepared hydrated and then brought to the desired size by sonication, and secondly, sonicated vesicles are homogenized by extrusion through a polycarbonate membrane. The mixture of vesicles forming ingredients, phospholipids, and surfactant was dissolved in volatile organic solvent (chloroform-methanol), the organic solvent evaporated above the lipid transition temperature using rotary evaporator [3]. Final traces of solvent were removed under vacuum overnight. The deposited lipid films were hydrated with buffer (pH 6.5) by rotation at 60 rpm for 1 h at the corresponding temperature 10. The resulting vesicles were swollen for 2 h at room temperature. To prepare small vesicles, resulting LMVs were sonicated at using a probe sonicated at 40 ºC for 30 min 11. The sonicated vesicles were homogenized by manual extrusion 10 times through a sandwich of 200 and 100 nm polycarbonate membranes [4].

Procedure for Preparation of Placebo: The placebo formulation has been prepared by incorporating an ethanolic solution of soya phosphatidylcholine and sodium cholate in 2 ml mixture of methanol and chloroform in the ratio of 1:1. To prepare this, Soya phosphatidylcholine is melted in 1 ml ethanol, and 25 mg of sodium cholate is added [13]. The resulting mixture is added to 20 ml mixture of methanol and chloroform in the ratio of 1:1 in a 250 ml round bottom flask.

The flask then was attached to rotary evaporator heat is introduced (40 ºC), and the solvent is removed with the help of a vacuum until a thin film is obtained. The resulting film was then dried overnight and then hydrated using phosphate buffer (PH 6.5) for one hour in the rotary evaporator. This will give a milky colloidal or suspension system. The resulting suspension is then sonicated using a probe sonicator for 20 min at 40W and then centrifuged at high RPM (10000 RPM) for 1 h for complete phase separation. The vesicles obtained were then characterized for particle size by Malvern zeta sizer [5].

Characterization of Fistein:

Melting Point Determination: The melting point of fisetin was assessed using melting point apparatus (Scientific Apparatus, Delhi, India) by following capillary tube method [18]. One end closed capillary tube loaded with the drug sample was kept in capillary holder for observing melting point by melting point apparatus. The thermometer of the apparatus displayed the temperature at which the drug started melting [6].

Differential Scanning Calorimetric Study: DSC measurement of fisetin was carried out by using differential scanning calorimeter Instrument (Pyris 6 DSC, Perkin Elmar, USA) [7]. Fisetin sample (5 mg) was transferred to an aluminium DSC pan and sealed to execute the analysis by heating the sample from 30 to 300 ºC with a heating rate of 10 ºC/min. The data analysis was performed by using software Pyris (Perkin-Elmer, USA)

Thermal Behaviour Study: The excipients and drug interaction of formulations were assessed by DSC instrument (Pyris 6 DSC, Perkin Elmar, USA).

The pure fisetin sample and a lyophilised sample of optimized formulations (transethosomes and ethosomes) were kept in separate aluminium pans, and study was conducted between 40-400 ºC with a heating rate of 10 C/min under nitrogen atmosphere (20 mL/min). An empty pan maintained at an inert atmosphere under nitrogen purging served as reference [8].

UV Calibration Curve of Fisetin: The standard stock solution of fisetin was prepared by dissolving 10 mg of fisetin in 50 ml of methanol taken in 100 ml volumetric flask [9]. The solution was subjected to sonication for efficient dilution and made up to 100 ml with methanol. From the standard stock solution, aliquots of specific quantities were transferred to a series of 10 ml volumetric flasks, and volumes were made up to 10 ml with methanol. Absorbance values of each solution were noted against blank methanol at 362 nm. The calibration

curve was made by plotting the absorbance against concentration.

Partition Coefficient Estimation: The partition coefficient of fisetin was estimated in octanol/water at 37 ºC. The graduated tubes were filled with water and octanol (50 ml each). This was followed by the addition of 20 mg of fisetin in each flask and mechanical shaking of mixtures for 24 h. The obtained mixture was then transferred to a separating funnel and kept aside for 6 h to get it equilibrated. Then the separated octanol and water were taken in separate flasks, and the quantity of fisetin retained in the octanol was measured by UV spectrophotometer at 363 nm [10]. The following formula was utilized for partition coefficient estimation.

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

Where Ca = Ct- Ca, Ct is total fisetin concentration taken (20 mg), Ca is total fisetin concentration aqueous phase.

Fourier Transmission Infra-red (FTIR) Spectroscopy Study: The KBr pellet method was utilized to make FT IR spectra of fisetin using the Jasco-4100 FTIR system. The spectral analysis was carried out in the wavenumber range of 4000-400 cm^{-1}. The obtained spectra of samples were analyzed with respective peaks and wavenumbers and compared with that of reference spectrum [11].

High-Performance Liquid Chromatography Method: The HPLC analysis of fisetin was carried out by an HPLC system attached with an LC-20AT pump, an SPD-20A variable wavelength detector, endowed with Agilent LC Ezchrome software, and a rheodyne injection valve having 20 ml loop size [12].

Formulation and Evaluation of Dermal Vesicular Formulations:

Preparation of Vesicular Formulations: Transferosomes were prepared by the conventional rotary evaporation sonication method described by Gregor Cevc. The ethanolic solution of phospholipid and surfactant was taken in a clean, dry, round bottom flask. The drug solution in 20 ml mixture of methanol and chloroform in the ratio of 1:1 was added it. The organic solvent was removed by vacuum rotary evaporation above the lipid transition temperature [34].

Final traces of solvent were removed under vacuum overnight. The deposited lipid film was hydrated with drug solution in saline phosphate buffer (PBS) (pH 6.5) by rotation at 60 rpm for 1 h at room temperature. The resulting vesicles were swollen at room temperature to get large multi-lamellar vesicles (LMLVs). To prepare smaller vesicles, LMLVs was probe sonicated at 40 ºC at 40 W for less than 30 min 13 as shown in **Table 1** & **2.**

TABLE 1: FORMULATION COMPOSITION

S. no.	Name of formulation	Sonication time (min)	Drug (mg)	SPC	Surfactant
1.	FM 1	20	50	100	20
2.	FM 2	20	50	100	25
3.	FM 3	20	50	150	30
4.	FM 4	20	50	150	20
5.	FM 5	20	50	200	25
6.	FM 6	20	50	200	30

TABLE 2: FORMULATION DATA FOR VESICLE SIZE AND %EE

S. no.	Name of formulation	Vesicle Size (nm)	%Entrapment Efficiency
1.	FM 1	45	45
2.	FM 2	40	55
3.	FM 3	35	56
4.	FM 4	42	74
5.	FM 5	64	83
6.	FM 6	42	61

Effect of Sonication on the Vesicle Size: To have the optimal vesicle size (100-200) the prepared optimized formulation was ultrasonicated for a specific time and effect of time to produce the optimum ratio was studied [14] AS shown in **Table 3.**

Drug Entrapment Studies (% Entrapment Efficiency Studies): The concentration of fisetin in the formulation was determined by HPLC analysis after disruption of the vesicles with Triton X-100 (0.1% w/v) at a 1:1 volume ratio and appropriate dilution with PBS (pH 7.4). The vesicle/Triton X-100 solution was centrifuged at 10,000 rpm at 4 ºC for 10 min. The supernatant was filtered with a 0.45 m nylon syringe filter [15]. The entrapment efficiencies of the paclitaxel-loaded formulation were calculated by using the formula given below

Percentage entrapment = (Drug entrapped (mg)/(total drug) × 100

TABLE 3: EFFECT OF SONICATION ON VESICLE SIZE AND ENTRAPMENT EFFICIENCY DETERMINATION OF DEVELOPED FORMULATIONS

S. no.	Name of formulation	Sonication Time(min)	Drug Lipid (mg)	Drug Content (mg)	Surfactant (mg)	Vesicle size (nm)	Entrapment Efficiency %
1.		15	150	50	30	180	70
2.	FM 5	20	150	50	30	62	82
3.		25	150	50	30	55	35

Optimization of Transethosomes: The optimum formulation of fisetin-loaded transferosomes systems was selected based on the criteria of attaining the maximum value of percent entrapment efficiency and small vesicle size. The FM5 was found to be the best formulation that has been fulfilled the requisites of an optimum transfersomal formulation [16].

Formulation of Transfersomal Gel: To prepare the transfersomal gel 1.5 g of carbopol 940 was added into 100 ml water in a beaker and was stirred for 24 h using a magnetic stirrer.

The swelled gel was then treated with triethanolamine solution (0.5% w/w of triethanolamine) to neutralize Carbopol-940 and to get a transparent gel structure. Transferosomes and carbopol 940 gel then mixed in the ratio of 1:1 w/w to form transfersomal gel [17].

Method of Preparation of Gel: Gel was prepared by the cold mechanical method described by Schmolka. Required quantity of polymer (Carbopol 940 & Bromopol) was weighed, and it was sprinkled slowly on the surface of purified water for 2 hrs. After which, it was continuously stirred by a mechanical stirrer till the polymer was soaked in the water.

With continuous stirring, diethanolamine was added to neutralize the gel, and it maintains the pH of the gel. Now the appropriate quantity of cremophor and PEG were added to the gel for enhancing the solubility of the drug, which behaves as the penetration enhancer, followed by the required quantity of isopropyl alcohol AS A preservative. Finally, the drug fisetin was added to the gel with continuous stirring till the drug get dispersed in the gel completely [18].

Characterization of Gel:

Extrudability: The extrudability was measured by observing weight in grams applied on a collapsible tube containing gel formulation to extrude the gel content of 0.5 cm within 10 seconds [19].

Spread Ability: This is generally expressed as areas covered on the application site, such as skin by gel on accounts of its spreadability characteristics. A glass plate pre-marked with a circle having 2 cm area was taken. The gel formulation weighed in 0.5 g gram was cautiously placed in that circle, and another second glass plate was placed upon the first glass plate. Then the weight of 500 g was put upon the upper glass plate and left as such for 5 min [20]. The spreading of gel in between the glasses will lead to an increase in diameter, which is noted. The % spread by area was calculated as follows

% Spread by area = (A2/A1) 100

Where, A1 = 2 cm original area and A2 = Area obtained after spreading.

Drug Content: The drug content of the gel formulation was measured by the HPLC method by appropriately dissolving 1 gram of gel in 100 ml methanol taken in a volumetric flask. The measurement of each was taken in triplicate and was expressed AS percentage of drug content [21].

***In-vivo* Studies:** The photocarcinogenesis protocol used in for UV-induced tumour initiation and

promotion to observe the photo protective potential of fisetin loaded ethosomal gel. The mice were divided into three groups, each composing eight mice. The first group served AS normal control; the second group was UV control, and the third group treatment group. The mice in groups two and three were irradiated with UV radiation on a daily basis for a period of 10 days at 180 mj/cm2. This exposure is intended for the initiation of tumours in mice. The last exposure of UV radiation was followed with one week break, and again they were exposed to UVB radiation (180 mj/cm2) with an average of three times a week induce tumour promotion [22].

This time the mice in group three were treated with fisetin-loaded ethosome gel at a dose of 150 µg before 15 min of each exposure. During this period, they were observed weekly to find out any possible tumors. The tumours observed in mice were recorded until their size gets stabilized, and their dimensions were also noted. The tumour volumes were estimated by following hemi ellipsoid model formula.

$$\text{Tumour volume} = 1/2(4\pi/3)\ (1/2)\ (w/2)\ h,$$

Where l, w and h denote tumour length, width, and height, respectively.

The incidence of carcinoma and their multiplicity were recorded weekly up to the 30^{th} week of experiment completion. The incidence of carcinoma was confirmed by a histological examination conducted on the mice either who died during the experiment or after the experiment's termination on 30^{th} week.

Skin Irritation Study: The mice were conditioned by removing hairs from their dorsal surface with hair-removing cream. The animals were separated into three groups containing three mice in each. Group I was considered as control, and group II and III were treated with conventional gel and transferomal gel of fisetin. The mice were topically applied with one gram of gel daily for seven days [23]. The mice were analyzed visually every day for the appearance of any erythema after 24 h of formulations treatment. The observations were recorded and matched with a scoring scale ranging from 0 to 4 AS given below in **Table 4.**

TABLE 4: EVALUATION OF SKIN IRRITATIONS

Record	Observation
Score 0	Non
Score 1	Slight (light pink color)
Score 2	Moderate (dark pink color)
Score 3	Moderate to severe (dark pink color)
Score 4	severe(dark red color)

Stability Studies: The stability studies of optimized fisetin transferosomal formulations were carried by analyzing their physical or chemical characteristics during storage. The stability studies were performed in accordance with the ICH guideline. 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 ºC and 25 ± 2 ºC /60 ± 5% RH. The following parameters were analyzed for the formulations at specific time periods of 0, 1, 3, and 6 months [24].

Visual Observation: Transparency, translucency, clarity, and homogeneity of the formulations were assessed by visual observations [25].

Mean Particle Size Analysis: Size distribution profile was determined by dynamic light scattering based on laser diffraction method employing Nanotrac Particle Size Analyzer (Nanotrac NPA250, Microtrac Inc., York, PA, USA). The experiment was performed in triplicate [26].

Drug Entrapment Efficiency and Loading Capacity: The clear supernatant was separated carefully and analyzed by UV spectrophotometer at 327 nm for OZ content after appropriate dilution with phosphate buffer pH 7.4. This indicates the amount of free drugs. The liposomal pellet was re-dispersed in phosphate buffer pH 7.4, lysed by the addition of 2% Triton X-100 and sonicated for 10 min. The concentration of OZ was determined after appropriate dilution in phosphate buffer pH 7.4 at 327 nm using a UV visible spectrophotometer [27]. The value of drug entrapment efficiency and loading capacity of OZ in each formulation was calculated as follows:

$$\%\ \text{Entrapment efficiency (\% EE)} = QL/QT*100$$

$$\text{Loading capacity (LC)} = QL/L$$

Where, QL - Amount of OZ measured in the liposomes, QT - Initial amount of OZ added in

liposomes L- Weight of lipid in the liposome formulation.

Viscosity: The viscosity of the prepared gel formulations was measured with the help of Brookfield R/S plus cone and plate rheometer with spindle RPM and 25 ± 2 ºC.

RESULT AND DISCUSSION

Preformulation Studies: The particle size range for all formulations of transfersomal fisetin gel was less than 200 nm (46 to 100 nm) with a narrow size distribution. The particle size range of the fisetin gel was significantly larger [28]. Out of six tranfersomal formulations, the best formulation was selected on the basis of particle size and % entrapment efficiency [29]. The FM5 tranfersomal formulation was found to be best, having a particle size of 62 nm and % entrapment efficiency of 82%. The vesicles containing cholesterol had a slightly lower particle size than without cholesterol. It was yellow-colored odorless fine powder.

Characterizations:

Differential Scanning Calorimetry (DSC): The DSC thermogram created for FIS and optimized transfersomes formulations are shown in **Fig. 1.** sharp endotherm observed at 321.3 ºC indicates the purity of the drug sample. The FIS's reported melting point is 330 ºC.

The FIS sample has shown a characteristic endotherm peak at 321 ºC. The characteristic endothermic peak of FIS at 321 ºC was found to be shifting to 167.54 ºC and 166.93 ºC for optimized formulations of transethosom and ethosome, respectively.

This could be due to the embedment of FIS inside the host lipid vesicle lattice. Because the embedment of guest molecules inside the host leads to shifting or disappearance of the melting, sublimating, or boiling temperature of guest molecules. This also suggests the alteration of lipid bilayer packing and their fluidization [30].

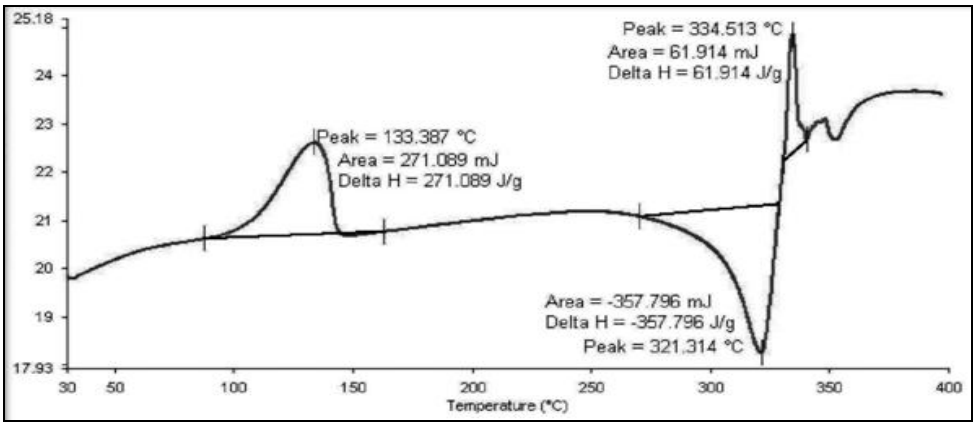

FIG. 1: DIFFERENTIAL SCANNING CALORIMETRY (DSC) FISETIN

Calibration Curve in Phosphate Buffer Ph 7.4: Standard solutions of different concentrations were prepared and their absorbance was measured at 260.4 nm, 222.4 nm, respectively, for fisetin [31]. Drug concentrations versus absorbance curves were plotted as given in **Fig. 2** & **Table 5.**

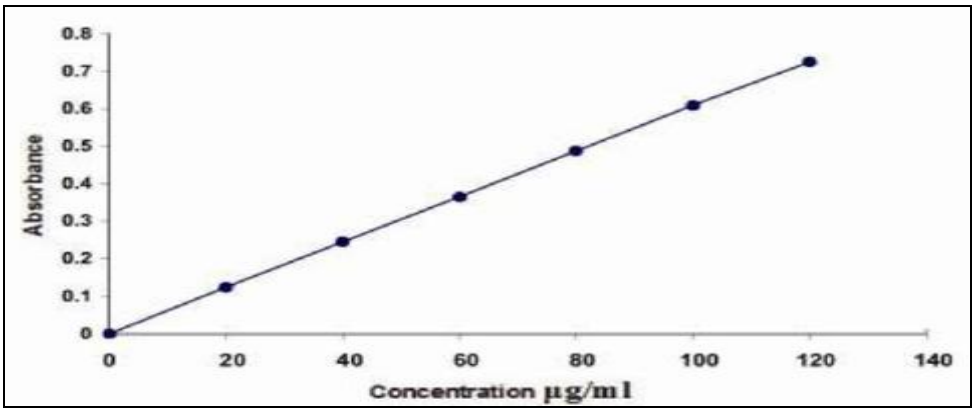

FIG. 2: CALIBRATION CURVE OF FISETIN

TABLE 5: CALIBRATION DATA OF FIESETIN IN PBS PH 7.4 AT 260.2 NM

Concentration (µg/ml)	Absorbance
0.0	0.0000
25	0.1242 ± 0.05
35	0.2447 ± 0.05
45	0.3638 ± 0.03
55	0.4865 ± 0.03
65	0.6087 ± 0.02
75	0.7249 ± 0.03

UV-Spectral Analysis: The observed λ_{max} of FIS (362 nm) methanol was found to be similar to that of the reported λ_{max} of 362 nm. The UV spectrum of FIS is shown in **Fig. 3.**

Partition Coefficient: The estimated partition coefficient of the fisetin sample at 37 ºC was 3.35, which was in close proximity to the reported value of 3.2.

FTIR Study: FTIR study of drug samples was carried out to confirm the purity of FIS drug samples. The main functional groups present in the FIS chemical structure are -OH, Ar C=C, C-O, C=O, C-C, apart from the aromatic ring structure [32]. The IR spectrum obtained for the FIS sample is shown in Figure. All these major functional groups have clearly displayed their infrared absorption peaks at their designated regions [33]. The observed IR absorption peaks are 3517.78 cm^{-1} and 3347.25 cm^{-1} (O-H groups), 1522.28 cm^{-1} (Ar C=C), 1112.08 cm^{-1} and 1017.45 cm^{-1} (C-O), 1603.34 cm^{-1} (C=O), 1569.38 cm^{-1} (C-C) as shown in **Fig. 4.**

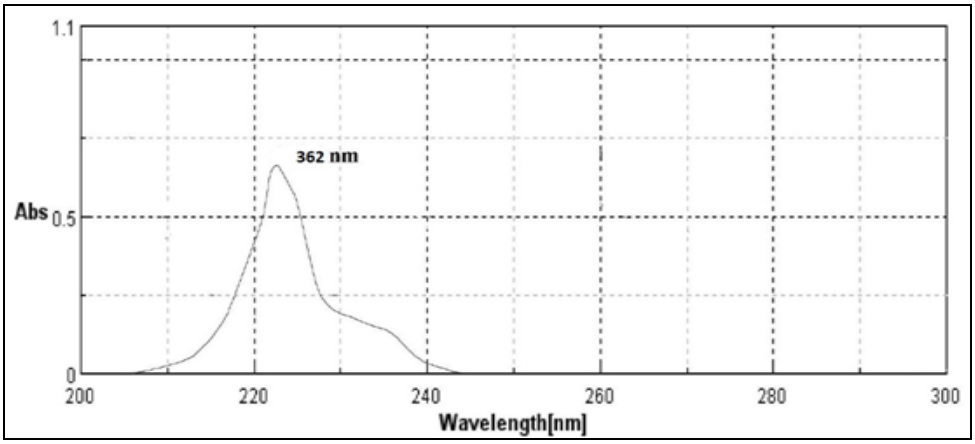

FIG. 3: UV SPECTRUM OF FISETIN

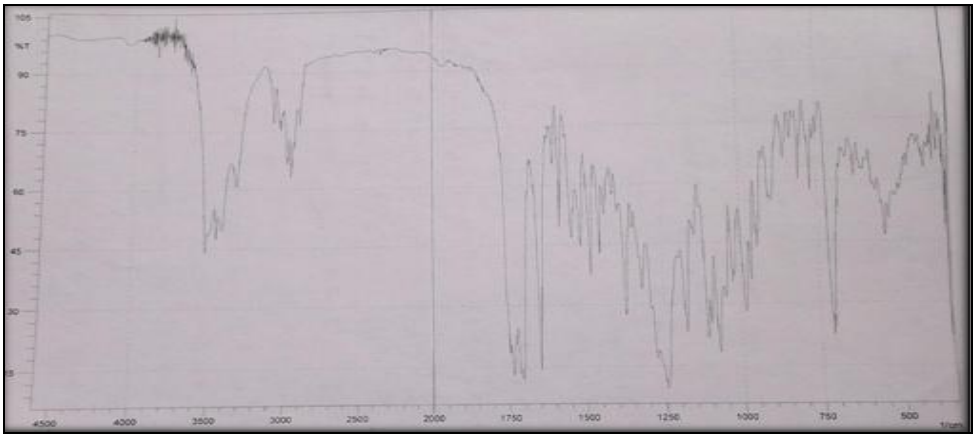

FIG. 4: FTIR STUDY OF DRUG

***In-vivo* Studies:** The figures shown in the permeation study illustrate the permeation profiles of fiesetin conventional formulations and paclitaxel -loaded transfersomes [34]. The cumulative amount of drug increased linearly with time after a short lag time (1-24 h). This linear accumulation was also observed for other formulations. The flux (F) of fisetin through the rat skin was calculated from the permeation profiles. The Flux of fisetin permeated through the skin in all vesicle formulations was significantly higher than the fisetin conventional formulations [36]. The vesicle systems were able to promote skin permeation of an active drug by a variety of mechanisms: (a) the

free drug mechanism, (b) the penetration-enhancing process of the transfersome components, (c) vesicle adsorption to and/or fusion with the Stratum Corneum and (d) intact vesicle penetration into and through the intact skin and the localization at the site of action [37].

Moreover, the similar predominance to the lipid bilayer of biological membranes and the nanometer size range of the vesicles may also be influenced.

These results indicated that the vesicle system can overcome the barrier function of the stratum corneum by various mechanisms and their physicochemical properties [38]. The drug is linear at 20 to 100 g/ml concentration, and the regression equation obtained from the data is given below.

$$Y= mx +C$$

Where Y=AUC, m =s lope (575561), X = conc. (µg/ml), C = intercept (-4E06 in this equation)

Stability Studies: The accelerated stability studies were carried out in accordance with the ICH guidelines. The ability of vesicles to retain the drug was assessed by keeping the transfersomal suspension at different temperatures. Optimized transfersomal formulations were selected for stability studies of vesicles. When transfersomes were observed under the microscope, there was no significant physical change or increase in average vesicle size was observed [39]. As they were insignificant, data are not reported in the thesis. Since the drug's stability and vesicles' stability are the major determinants for the stability of formulations, studies were carried out to evaluate total drug content and drug entrapment at room temperature (30 ± 2 ºC) and refrigeration temperature (4 ± 2 ºC). Stability study could not be carried out at higher temperature (>60 ºC) because phospholipid used as one of the major components for transfersomes would get deteriorated at higher temperature [40]. Hence from the study, it can be concluded that transfersomal formulations containing 30% ethanol concentration can be considered highly stable than the rest AS shown in **Table 6.**

TABLE 6: STABILITY STUDIES OF VESICLES

Formulation Code	% Entrapment Efficiency				
	Initial	After 2 Weeks	After 4 Weeks	After 6 Weeks	After 8 Weeks
FET 1	50.83 ± 0.42	50.53 ± 0.39	47.41 ± 0.84	49.02 ± 0.81	43.61 ± 0.57
FET 2	57.51 ± 0.44	57.02 ± 0.61	54.52 ± 0.36	55.81 ± 0.39	52.40 ± 0.39
FET 3	32.77 ± 0.39	31.26 ± 0.81	27.28 ± 0.57	28.27 ± 0.61	23.22 ± 0.61

CONCLUSION: This study concluded that the developed, validated method is statistically significant. The degradation study results show that the drug is stable at alkaline and oxidative conditions [41]. This method proved to be simple, accurate, precise, specific, and selective [42]. Hence the method thus developed is recommended for routine studies in the industries.

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DECLARATION OF INTEREST: The authors report no conflicts of interest.

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