# FORMULATION AND EVALUATION OF NANOLIPOSOMES CONTAINING ISOLIQUIRITIGENIN

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**Abstract**

Psoriasis is a chronic inflammatory skin disease that attack the immune system. Isoliquiritigenin is one of the most important bioactive compound derived from licorice root. However, its therapeutic use is restricted due to its poor aqueous solubility and limited bioavailability. To overcome these limitations, a novel ILG-loaded nanoliposomes (ILG-NL) is designed. The aim of the present study was to evaluate the nanoliposomes containing isoliquiritigenin for its anti- psoriatic action. The different polymers at various ratios were used to formulate the formulations. The formulations were prepared by injection method. The prepared formulations were evaluated for different parameters including particle size, PDI, zeta potential, drug loading and entrapment efficiency. After primary evaluation formulations F3 was selected for further studies including drug release, HRTEM and antipsoriatic potential. The nanoliposome (F3) was found to be superior in the in-vitro release testing and possess better antipsoriatic potential.

**Keywords:** Isoliquiritigenin, psoriasis, nanoliposomes, analytical method development, validation

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## Introduction

Psoriasis is a chronic inflammatory skin disease that attack the immune system. It is autoimmune disorder where skin cells were considered as a foreign substantial which leads to triggers falls signal that speeds the growth cycle of the skin. There is formation of thick silvery surface, dry red patches occur due to rapid accumulation of skin cells. Nearly 2- 5% of world population is affected by this disease condition[1].

Isoliquiritigenin (2′,4′,4-trihydroxychalcone, ILG), one of the most important bioactive compounds with a chalcone structure, is derived from licorice root. Licorice is commonly known as Glycyrrhiza. Liqorice have soothing effect on skin and helps to ease inflammation. It helps to reduce redness, irritation and swelling and used to treat conditions like dermatitis and eczema. Isoliquiritigenin offered anti-cancer activity[2,3], anti-inflammatory action[4], hepatoprotective action[5,6] against D-galactosamine lipopolysaccharide (GalN/LPS)-induced toxicity.

Nanoliposomes are nanosized version of liposomes, which are widely used encapsulation and control release system[7]. The prime ingredient of liposomes are lipids and/or phospholipids. Phospholipids are amphiphilic having both hydrophobic and hydrophilic groups. The head group is hydrophilic, and the tail is a fatty acid chain which is hydrophobic[8]. Cholesterol is widely used sterols in manufacture of liposomes. Cholesterol imparts stability to liposomes by modulating fluids of lipid bilayer.

Nanoliposomes also improve the solubility and bioavailability, as well as stability both in vitro and in vivo. The added advantage of nanoliposomes is cell targetability. This is an important characteristic to attain significant drug concentration at target site thereby minimizing adverse effects on healthy cells[9]. Liposomes offered various advantages viz; liposomes deliver both hydrophobic and hydrophilic drugs, improved stability via encapsulation, improved medication potency as well as therapeutic efficacy, in case of systemic and non-systemic doses, liposomes are non-toxic, flexible, biocompatible, totally biodegradable, and non-immunogenic, liposomes assist decrease the exposure of sensitive tissues to hazardous medications by reducing the toxicity of the encapsulated agent. No study was reported on isoliquiritigenin for its antipsoriatic action. Therefore the present study was performed to evaluate antipsoriactic action.

## Material and Method

### Material

Isoliquiritigenin is purchased from Yucca. All the other chemicals were procured from S. D. Fine Chem. Ltd Mumbai.

### Method

### Development and Validation of HPLC method to Evaluate Isoliquritigenin

### HPLC Method Development [10, 11]

The chromatographic separation was performed using Agilent Tech. (1100) system. The Fortis C18 (100 x 4.6 mm id with 2.5µm particle size) column was used to separate the isoliquritigenin. Different solvents including acetonitrile, water were tried for the separation of components. The effect of flow rate on the separation of component was evaluated by setting flow rates at different values ranging from 0.5 to 1.5 ml/min. The UV spectrophotometer was used to perform a scanning of solution between the wavelength range of 400-200 nm. The peak area was calculated with the software CHEMSTATION 10.1 to arrive at an estimate of the quantity of the medication.

### Preparation of mobile phase

HPLC grade acetonitrile and water were combined in a 75:25 v/v ratio along with 0.1% of OPA to formulate the mobile phase. The material was passed through a 0.45 µm membrane filter after being sonicated for 15 minutes. Degassed the mixed solvents and utilized as the mobile phase.

### Preparation of reference standard and sample solution

Isoliquiritigenin is used as standard. The standard was prepared by using 10 mg of standard in 10 ml of methanol. The formulation equivalent to 10 mg used to prepared sample solution.

### Method validation parameters

The validation of the developed HPLC method was carried out in accordance with ICH guidelines. The linearity was analysed for concentration ranging from 5- 100 µg/ml by using Least-square regression analysis where, peak areas were plotted against the corresponding concentrations. The intra- day and inter- day precision and repeatability was evaluated by triplicates of three different concentrations of each isoliquiritigenin was spotted and analyzed on same day for intra-day study and two different days for inter-day study with respective chromatographic conditions.

Recovery study method was employed to evaluate accuracy. The samples were spiked with 80, 100 and 120 % of median concentrations of standards.

$$\text{Accuracy} = \frac{\text{spiked concentration} - \text{mean concentration}}{\text{spiked concentration}} \times 100$$

Robustness was carried out by making deliberate changes in the wavelength, flow rate and mobile phase and evaluated their effect on the retention factor. The estimation of LOD and LOQ were done by standard deviation method. Detection limit =3.3σ /S and quantitation limit=10 σ /S (σ is residual standard deviation of a regression line and S is the slope of the calibration curve).

**Compatibility Study**

**Fourier transform infrared spectroscopy**

To record Fourier transform infrared spectroscopy (FTIR) spectra of the formulation prepared by melt dispersion technique with conjugate (FM1.1), a FTIR spectrometer (FTIR-8400; Shimadzu Corporation, Kyoto, Japan) equipped with a diffuse reflectance accessory (DRS-8000; Shimadzu Corporation, Japan) and a data station was used.

**DSC**

The sample prepared by melt dispersion technique with conjugate was evaluated for thermal changes. The evaluation was performed on DSC 25 Mettler (Perkin-Elmer). The 5 mg of formulation prepared by melt dispersion technique with conjugate was utilized for evaluation.

**Formulation by injection method**

The formulations were prepared by dissolving drug with phospholipid (Lecithin) and amount of cholesterol in different predetermined ratios and dissolved in the ethanol and chloroform mixture (1:1) as mentioned in Table 1. Briefly, different ratios of Drug: Lecithin (1:2 to 1:8) and Lecithin: cholesterol (2:1) was taken and dissolved in ethanol: chloroform (1:1) mixture. The volume of ethanol and chloroform was increased proportionally as the lipid content in drug: lipid ratio was raised. By using ultrasonic waterbath the mixtures were then sonicated for 15 min. The homogenous solutions were then added quickly using the syringe to 25ml deionized water previously stirrer at 500 RPM on magnetic stirrer using teflon-coated bead. The stirring was continued until the chloroform and ethanol got evaporated. Each composition was stirred between time ranges of 4-8 hrs. The prepared liposomes were stabilized by keeping in the refrigerator for at least 6 hrs. The formulations ILGNL1 to ILGNL7 prepared by injection method are given in table 2.

**Table 1:** Drug to lecithin ratio in nano-liposome (NLs) formulations by injection method

| Sr. No. | Formulation Code | Ratio | | |
|---|---|---|---|---|
| | | Drug : Lecithin | Lecithin: Cholesterol | Ethanol: Chloroform |
| 1. | ILGNL1 | 1:2 | 2:1 | 1:1 |
| 2. | ILGNL2 | 1:3 | 2:1 | 1:1 |
| 3. | ILGNL3 | 1:4 | 2:1 | 1:1 |
| 4. | ILGNL4 | 1:5 | 2:1 | 1:1 |
| 5. | ILGNL5 | 1:6 | 2:1 | 1:1 |

**Table 2:** Composition of the nano-liposome (NLs) formulations by ethanol injection method

| Sr. No. | Name of Ingredients | ILGNL1 | ILGNL2 | ILGNL3 | ILGNL4 | ILGNL5 |
|---|---|---|---|---|---|---|
| 1. | ILG | 5 mg | 5 mg | 5 mg | 5 mg | 5 mg |
| 2. | Sunflower lecithin | 10 mg | 15 mg | 20 mg | 25 mg | 30 mg |
| 3. | Cholesterol | 5 mg | 7.5 mg | 10 mg | 12.5 mg | 15 mg |
| 4. | Ethanol and<br>Chloroform (1:1) | 2.5 ml<br>2.5 ml | 3.75 ml<br>3.75 ml | 5 ml<br>5 ml | 6.25 ml<br>6.25 ml | 7.5 ml<br>7.5 ml |
| 5. | Deionised water | 25 ml | 25 ml | 25 ml | 25 ml | 25 ml |

**Characterization of ILG-loaded nanoliposomes**[12- 15]

**Particle size**

At a temperature of 25°C, the Zetasizer device was used to measure particle size. The mean particle diameter and particle size distribution are produced using this method. Malvern Instruments software was used to do the analysis. Samples were kept in a refrigerator set at 4°C before analysis.

**Polydispersity Index**
Polydispersity index is indicative of uniformity in the particle size and hence it should be as low as possible. The measurement of PDI was obtained during the analysis of particle size.

**Zeta potential**
The formulation of nanoliposome was tested for zeta potential using Malvern Zetasizer instrument. Zeta potential was determined by zeta potentiometer. The sample was filled into the cell; an electrode inserted was placed under the microscope and connect them to the zeta meter. The analysis was carried out at 25°C.

**Drug loading**
HPLC was used to determine drug loading. Using deionized water, one ml of nanoliposome formulation was dissolved in 1 ml ethanol, resulting in a volume of 10 ml. After that, the solution was sonicated for 5 minutes. After that, 0.45 m filters are used to filter the solution. The filtrate was then subjected to HPLC analysis.

**Determination of Entrapment efficiency**
The nanoliposome formulation (10 µg/ml) was centrifuged at 4000 rpm for 18 min at 4°C temperature by using Remi cooling centrifuge. A supernatant contains the nanosponges in suspending stage and oil on the wall of centrifuge tube. The supernatant was again centrifuged at 15000 rpm for 30 min at 4 °C temperature. As a result, a transparent solution of supernatant and nanoliposome was attained which was redispersed in mobile phase (100 ml). The solution was further sonicated, the nanoliposomes were disrupted to discharge the isoliquiritigenin. The drug was determined for the drug entrapment. The amount of isoliquiritigenin was estimated by using HPLC system.

$$\text{Percentage Entrapment Efficiency} = \frac{\text{Wc}}{\text{Wt}} \times 100$$

Where amount of drug content (entrapped) in the nanoliposome is denoted as Wc and total amount of drug in the dispersion is denoted as Wt.

**In- vitro study**
*In-vitro* release study of isoliquiritigenin from the formulation (ILGNL3) was carried out in phosphate buffer pH 5.5 acetate buffer by the dialysis membrane method. The amount of formulations equivalent to 10 mg of isoliquiritigenin was taken in dialysis bags for pH 5.5 medium (by cutoff of 12,000 Da, Sigma). The drug dispersion and formulation containing dialysis bags suspended in a beaker with 100 ml of acetate buffer maintained at pH 5.5 was kept on a magnetic stirrer which is rotated at 100 rpm, with temperature adjusted to 37±0.5°C for a selected time intervals. A 5 ml sample was withdrawn for analysis and replaced with the same quantity of a fresh media. The samples was then filtered through 0.45µm filter. The samples were analyzed for drug release by determining absorbance using HPLC, the rate of isoliquiritigenin release obtained using the standard curve.

**Surface morphology**
The particle size and morphology of nanoparticles was examined by HRTEM (Tenai G2 20 Twin, FEI Company, Netherland).
Sample of nanoliposomes (ILGNL3) was prepared by taking a drop of sample on parafilm. Then, a drop of 2% phosphotungstic acid solution were kept over sample drop and left it for 30 sec. The copper grid was placed on sample. Then air drying of copper grids were performed for 1 h and observed under HRTEM and photomicrographs were captured.

**Anti-Psoriatic: Oxazolone mice model [16, 17]**
After the approval from the Institutional Animal Ethics Committee (Protocol No. PESRTBCOP/ IAEC, 2022R-96). The experimental work was performed at animal house of Rajaram and Tarabai Bandekar college of Pharmacy, Ponda, where Albino Wistar rats of 200-250 g weight were used to determined psoriatic action. The animals were divided in four groups. Group I as normal, group II as control, group III as ILG and group four as NL-ILG.

**Sensitization and Elicitation (Challenge Application) Procedure:** The animals were sensitised for six days by applying 100 µl of 1.5% oxazolone in ethanol to their abdomen area. On days 7, 10, 13, and 16, 20 µl of % oxazolone in a combination of acetone and olive oil (4:1) was applied to both sides of the mouse ear after 7days of sensitization.

**Measurements:** Ear thickness was estimated using digital Vernier Calipers at several time periods during the investigation. To determine swelling ear responses, ear thickness was evaluated before the sensitization phase (Day 7) and after each elicitation on days 10, 13, 16, and 19. Mouse ears were removed, fixed in 10% buffered formalin solution, embedded in paraffin, cut into 5 µm slices, and stained with hematoxylin-eosin 72 hours after the last administration of oxazolone using conventional techniques. The epidermal

thickness was evaluated as the distance between the bottom of the stratum corneum and the foundation membrane in the interfollicular epidermis during the histological examination, after the microscopic fields were photographed. Inhibition of epidermal thickness was calculated.

## Results and Discussion

### Development and validation of HPLC method

### Optimization of RP- HPLC method

The optimization of the RP-HPLC chromatographic parameters were carried out by using different compositions of mobile phase and flow rates. The separation was carried out on Agilent Tech. (1100) system using acetonitirile: water (0.1% of OPA) in a ratio 75:25 v/v with the flow rate 1 ml/min as it gave well resolved peak. Based on peak area quantification was carried out at 355 nm. The Rt for isoliquiritigenin was found to be 9.245 min given in figure 1.

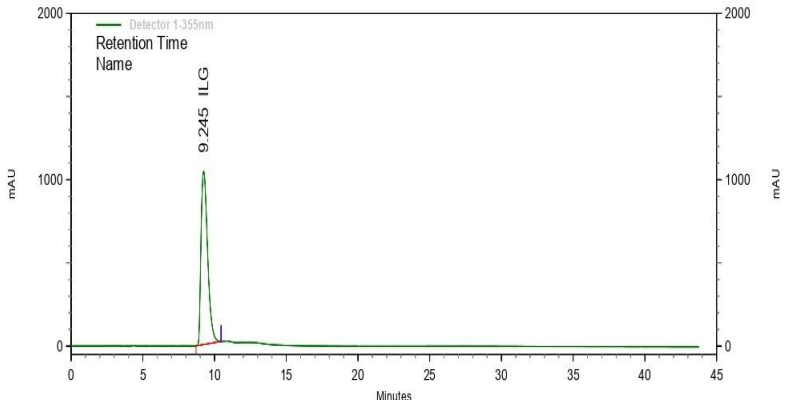



**Figure 1:** Chromatogram of isoliquiritigenin standard

### Method validation parameters

### Linearity

In order to ascertain the linearity of the method, the standard stock solution was diluted to yield concentration ranges of 5 to 100 µg/ml. The results demonstrate an outstanding correlation between peak area and analyte concentration. By plotting the AUC versus the analyte concentration, the calibration curve was constructed and analysed using linear regression (Figure 2).

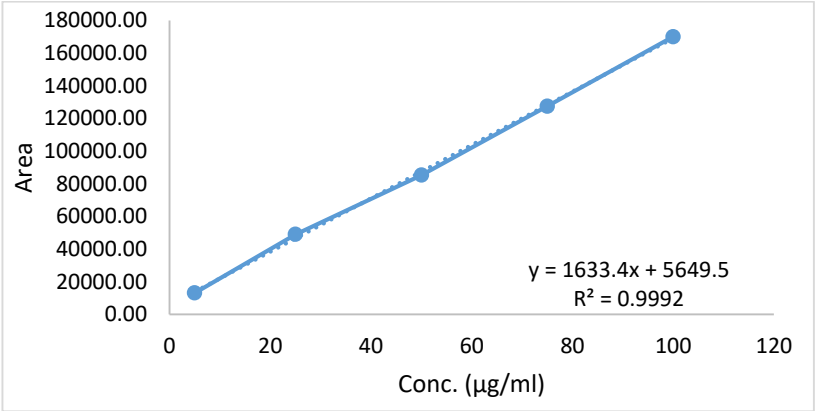



**Figure 2:** Linearity curve of isoliquiritigenin

### Accuracy

A successful drug recovery research at three different concentration levels showed that the method used was effective. Pre-analyzed samples were mixed with a known amount of a reference medication (80, 100, or 120%) before being put through the suggested HPLC procedure. The recoveries were found to be in the range of 101.24-101.76%. The % recovery was found to be within the limits.

### Precision

Data on repeatability and instrumental variation were obtained in triplicate. Method precision was evaluated by repeatedly introducing 50 µg/ml concentration of isoliquiritigenin. The developed method was found to be precise as % RSD was found to be 1.03. Intraday and interday precision was done in triplicate at 3 distinct concentration levels. The % RSD was found to be 1.16, 0.64 and 1.64 for interday precision and 0.75, 0.20, and 0.85

for intraday precision. RSD < 2%, proved that the method was highly precise.

**Robustness**

Robustness was done by small changes in the chromatographic conditions like mobile phase, flow rate and wavelength. It was observed that there were no marked changes in the chromatograms. The developed method was found to be robust as the % RSD values were< 2.0 %.

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**

The LOD and LOQ was found to be 1.26 µg/ml and 3.81 µg/ml respectively.

**Evaluation of formulation**

The prepared nanoliposomes were further studied for different parameters such as particle size, poly dispersibility, zeta potential, % drug loading and entrapment efficiency given in table no. 3, figure 3, 4.

**Table 3:** Evaluation of Nanoliposomes

| Sr. No. | Formulation | Particle size (nm) | PDI | Zeta potential (mV) | %Drug loading | % Entrapment efficiency |
|---|---|---|---|---|---|---|
| 1. | F1 | 274 ± 0.14 | 0.251 ± 0.033 | -27.75 ± 0.254 | 81.68 ± 1.47 | 83.62 ± 1.55 |
| 2. | F2 | 704.4 ± 0.51 | 0.751 ± 0.017 | 16.54 ± 0.521 | 84.25 ± 0.65 | 70.20 ± 2.91 |
| 3. | F3 | 211.2 ± 0.45 | 0.208 ± 0.021 | -45.26 ± 0.512 | 95.15 ± 0.80 | 90.61 ± 1.33 |
| 4. | F4 | 317.1 ± 0.63 | 0.661 ± 0.002 | 51.4 ± 0.262 | 80.17 ± 1.93 | 70.90 ± 1.05 |
| 5. | F5 | 834.0 ± 0.79 | 0.533 ± 0.003 | -32.2 ± 0.289 | 88.82 ± 0.55 | 74.53 ± 0.60 |

Polydispersity index is an indication of the size distribution with values ranging from 0 to 9. The Poly-dispersibility index ranges from 0.208 ± 0.021 to 0.751 ± 0.017. Particle size ranges from 211.2 ± 0.45 to 834.0 ± 0.79. The evaluation indicate that the formulation F3 is better as it showed small particle size and PDI. As smaller particle size indicates better diffusion and permeability and lesser value of polydispersity indicates narrow size distribution of particles in the dispersion.

Zeta potential measurement the physical stability of nanodispersed system and predicted for long term stability. The maximum standard limit for zeta potential is ± 30 mV. The formulation F3 showed better % drug loading and % entrapment efficiency 95.15 ± 0.80 and 90.61 ± 1.33 respectively. Therefore formulation F3 was selected for further studies.

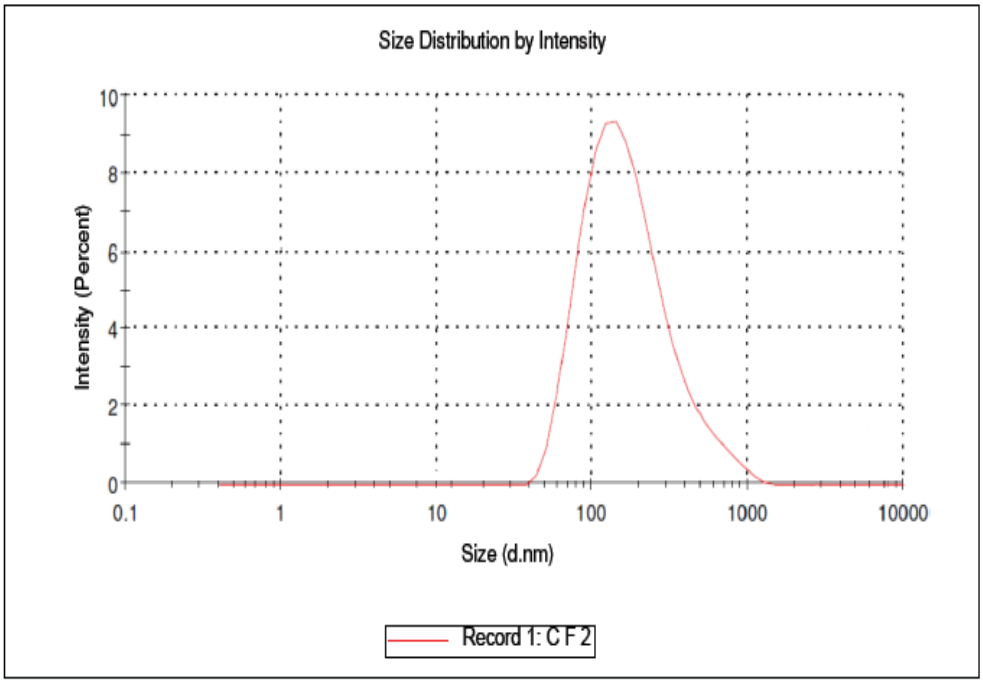



**Figure 3:** Particle size distribution of formulation F3

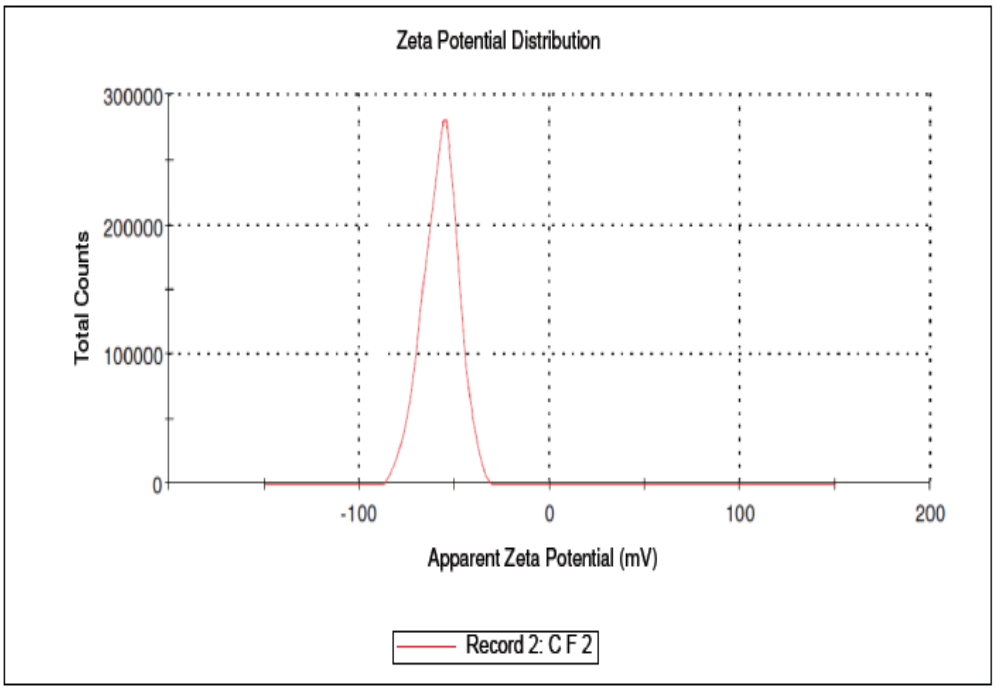



**Figure 4:** Zeta potential of formulation F3

**In vitro study F3**

The % drug release from nanoliposome formulation (ILGNL3) was found in first hour is 32.83%. At the end of 8 hrs around 93.13% drug was released from nanoliposome formulation (ILGNL3) given in figure 5. The nanoliposome prepared using PVA: EC (1:1) showed better release of drug from nanoliposome.

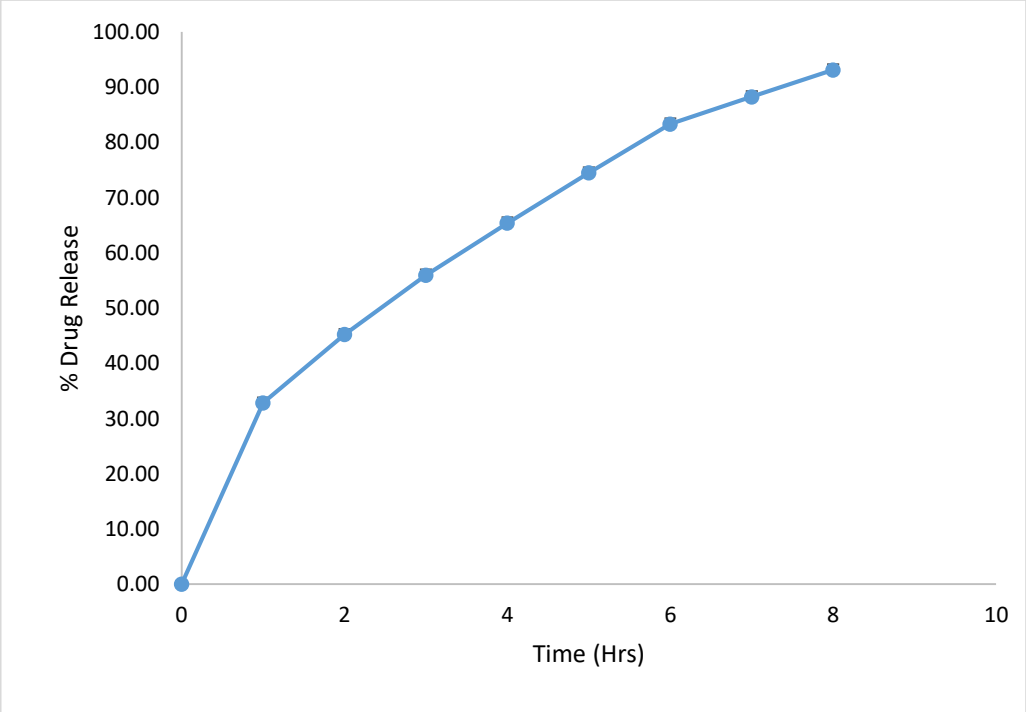



**Figure 5:** In-vitro study of nanoliposome formulation F3

**HRTEM**

The nanosponges prepared by using formulation F3 showed spherical (200 micrometer) with smooth surface, mono-dispersed pattern given in figure 6.

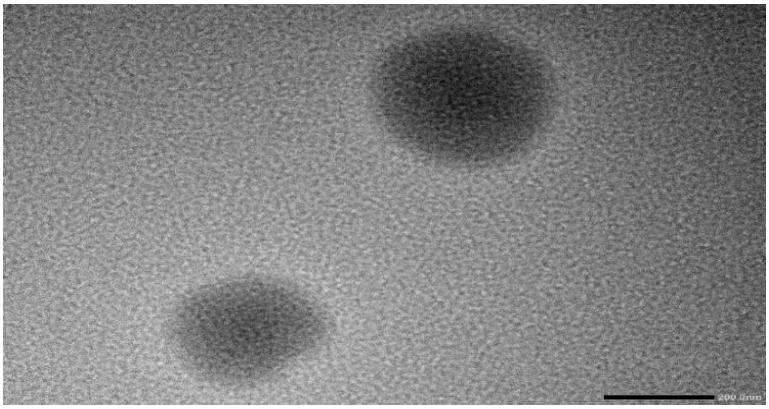


**Figure 6:** HRTEM evaluation of nanoliposome (F3)

**Animal Study**

The effect of formulation F3 was evaluated by topical application of formulation to oxazolone induced psoriasis in mice for 16 days. The animals were sensitized by applying oxazolone to the abdominal region of the mice. The formulation was applied after the challenge. The evaluation was performed by measuring the thickness of ear. After the completion of treatment with nanoliposome formulation showed significant decreased in ear thickness, indicating the anti-inflammatory potential of the nanoliposome formulation (F3).

The histopathology study revealed that, developed formulation of the active has increased the efficacy with decrease number of inflammatory cells, improved skin surface and reduction in the thickening of the skin.

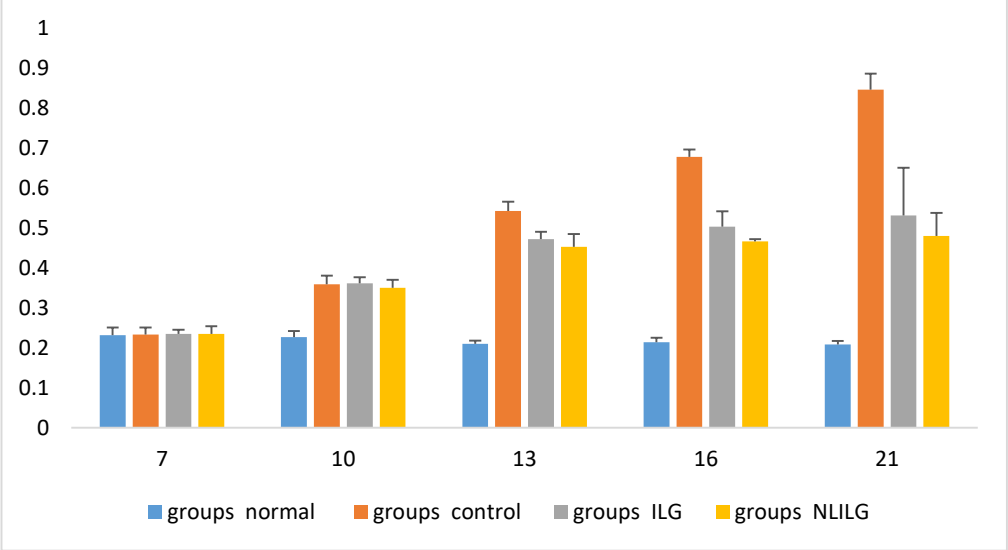
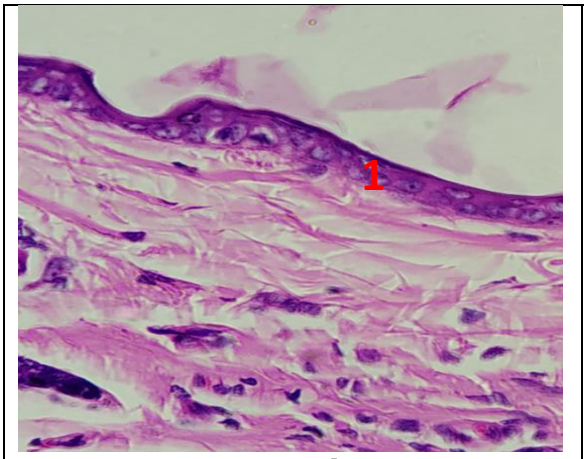
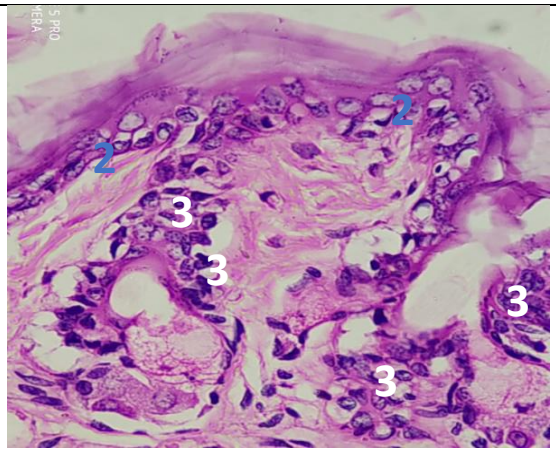



**Figure 7:** Determination of anti- psoriatic action of nanoliposome by measurement of ear thickness




**Normal**




**Control**

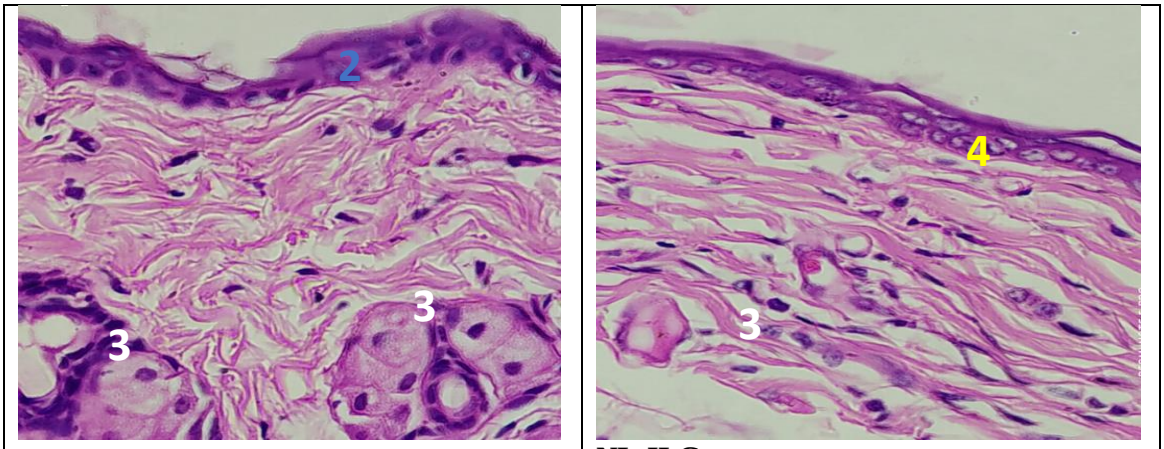



**Figure 8:** Histopathological study of skin in mice with oxazolone-induced psoriasis
* 1: normal epidermis,
2: thickened epidermis with psoriatic cells,
3: inflammatory cells,
4: keratinized layer

## Conclusion

Psoriasis is a chronic, non-communicable, painful, disfiguring, and disabling illness that reduces the quality of life. The aim of the present work was to prepared isoliquritigenin loaded nanoliposome and evaluated for its antipsoriatic activity. The different polymers at various ratios were used to formulate the nanoliposome (F1 to F5). The prepared formulations were evaluated for different parameters. After primary evaluation formulations F3 was selected for further studies including drug release, HRTEM and antipsoriatic potential. The nanoliposome (F3) was found to be superior in the in-vitro release testing and possess better antipsoriatic potential.

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**Conflict of interest** Authors declared no conflict of interest
**Author's contribution statements**

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