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# Development and Evaluation of Khelin-Loaded Ethosomes

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**ABSTRACT**

This study investigates the development and evaluation of khelin-loaded ethosomes for enhanced topical delivery. Ethosomes, lipid-based vesicles, are known for their potential to improve transdermal drug delivery by enhancing skin penetration. Using a thin-film hydration technique, khelin-loaded ethosomes were formulated and evaluated for particle size, zeta potential, encapsulation efficiency, and morphology. In vitro studies using Franz diffusion cells demonstrated a significant increase in khelin penetration and retention within the skin layers. Stability testing confirmed the ethosomes' durability under refrigerated conditions. The findings suggest that ethosomes are promising carriers for khelin, offering improved delivery and potential therapeutic efficacy in dermatological applications.

**Keywords:** Khelin, Ethosomes, Topical Drug Delivery, Skin Penetration, Encapsulation Efficiency

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## 1. INTRODUCTION

In recent years, ethosomes have gained popularity as carriers for enhancing the delivery of various therapeutic agents due to their unique structure and flexibility [1, 2]. This study aims to improve the penetration and efficacy of khelin, a molecule known for its therapeutic potential [3, 4]. Ethosomes, as lipid-based carriers, possess favorable attributes for enhanced skin penetration, as previously demonstrated in other drug delivery studies [5-7]. The preparation and characterization of ethosomal systems have been standardized through various methods (8-10). Moreover, the in vitro and ex vivo assessment techniques employed in this study, such as Franz diffusion cells, have been widely validated in topical formulation research [11, 12].

The ethosome formulations in this study-maintained stability under various storage conditions, supporting previous findings on ethosome stability in refrigerated conditions [13, 14]. Additionally, the skin deposition and permeation analysis suggest that ethosomes can improve the retention of therapeutic agents within the stratum corneum and epidermis, further supporting their utility in topical applications [15].

The goal of this research is to develop a khelin-loaded ethosome formulation, optimize its encapsulation efficiency and stability, and evaluate its in vitro release and skin penetration properties.

## 2. MATERIALS AND METHODS

### 2.1 Materials

- **Khelin**: sourced from a reputable supplier.
- **Phospholipids (e.g., Phosphatidylcholine)**: used for ethosome formation.
- **Ethanol**: acts as a penetration enhancer in ethosomal formulations.
- **Deionized Water**: used in all preparation and dilution steps.
- **Other Reagents**: Solvents for HPLC analysis, buffer solutions for in vitro studies.

### 2.2 Preformulation studies

#### 2.2.1 Physical Characteristics

The drug(s) sample shape, pH, odour, melting point, and other characteristics were noted.

**2.2.2 Tests for identification:**

**a) U.V. analysis**

Methanol was used to build up a volume of 10 ml and dissolve 1 mg of the drug(s). 5ml of the aforementioned solution were diluted to 100ml with methanol. 200 to 400 nm ranges were tested.

**b) Infrared Fourier Transform (FT-IR) Absorption Spectrum**

Khellin FT-IR spectrum was captured using the KBr pellet technique. The obtained spectra and the reference spectrum were compared (Nasr et al., 2022).

***c) Differential Scanning colorimetry (DSC)***

One milligram Khellin was carefully weighed and stored in side the aluminium DSC pan. The analysis was done at a temperature range of 20-350°C with a heating rate of 10°C/min. (Nasr et al., 2022).

### *2.3 Ethosome preparation*

The "Thin-film hydration method" was used to develop ethosomes using BBD (Design-Expert software, Version 13. The independent variable and the selected responses varied in each of the three situations (Table 1). Therefore, to develop a dry thin film, phospholipid and drug were dissolved in a chloroform and methanol (2:1) ratio in an RBF attached to a rotary evaporator (model used: HAHN SHIN, HS- 2005 V-N, Korea). This solution was kept at a low pressure. Phosphate buffer (pH 7.4) was used to rehydrate the film. A probe sonicator was used to size-reduce the coarse dispersion for 3-4 mins (Agarwal et al., 2018).

To obtain the free-flowing dry powder, the ethosomes underwent additional lyophilization. The materials were lyophilized for 36 hours after being frozen at -78°C for 10 hours. As required for the experiments, the lyophilized formulation was reconstituted (Agarwal et al., 2018).

### *2.4 Optimization of Formulation(s)*

The experimental design included five replicates (centre point) and twelve factorial experimental conditions used in the optimization. Randomization of the experimental runs was used to reduce the impact of unanticipated variability in the observed responses. To correlate the link between independent variables and responses, a second-order quadratic model was fitted.

**Table 1:** Variables coded and actual values for Khellin Loaded ethosomal formulations

| S. No. | Variables | Levels | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| 1. | Phospholipid (mg) | 10.00 | 15 | 20.00 |
| 2. | Ethanol (%) | 75.00 | 80 | 85.00 |
| 3. | Sonication time (mins) | 2.00 | 3 | 4.00 |

The relationship between the variables and the response was correlated. By measuring the coefficient ($R^2$) of determination, fitness evaluation was performed on the second- order polynomial model. The significance of the regression coefficients was assessed using an F-test and an ANOVA. The fitted polynomial equation was independently composed as a three-dimensional response to illustrate the link between the dependentvariable and independent factors.

### 2.4 Characterization of Developed Formulation(s)

#### *2.4.1 Size distribution analysis for vesicles*

Using a computerized inspection system (Nano ZS90, Malvern instrument, UK), the DLS method was used to assess the vesicle size. The size distribution parameter of choice was the polydispersity index (PDI). Before the size measurements, deionized water was used to dilute all vesicles 100 times. The determination was made three times. At 25°C, & particle sizes were measured (Cevc et al., 1992).

#### *2.4.2 Entrapment efficiency studies*

The UV technique was used to calculate the Khellin entrapment efficiency in vesicles. The free form of the drug and the integrated drug were separated using a centrifugation technique. The vesicle suspensions were centrifuged for 30 minutes and fifteen thousand rpm. Following centrifugation, the final solution and pellet have been separated and Khellin content were determined by UV method.

#### *2.4.3 Transmission ElectronMicroscope (TEM) and scanning electron microscopy (SEM)*

By using a transmission electron microscope (JEOL: 120CX Microscope, Japan) working at 120 kV, the morphology of the developed formulation was assessed. 1-2 minutes were given for the formulation to adhere to the carbon substrate after a drop was applied on a copper grid with a 300 mesh coating. 1%

phosphotungstic acid was dropped onto the grid, and the sample was left to air-dry. Finally, using soft imaging viewer software, the sample was scanned and viewed (El-Laithy et al., 2011).

The surface morphology of the ethosomal vesicle was studied using Scanning Electron Microscopy (SEM). On a transparent glass stub, one drop of the ethosomal system was applied, allowed to air dry, then coated with a Polaron E 5100 sputter coater (Polaron, UK) and seen using a Leo-435 VP scanning electron microscope.

### 2.5 In-Vitro Permeation *Studies*

Ethosomal formulation(s) were used to investigate the impact of ethosomal vesicles on skin permeability (El Sayed et al., 2006).

#### *2.5.1 Fabrication of diffusion cell*

A Franz diffusion cell was used, which was made by a nearby fabricator and included a water jacket to keep the assembly area temperature at 37°±0.5°C. It was composed of two half-cells. The donor compartment was on top, while the receiver compartment was situated below. The diffusion cell's effective area was 0.569 $cm^2$ and its receiver capacity was 15 ml. Using a recirculating water bath to keep the diffusion cell at 37°±0.5°C, the chamber solution was continuously stirred at 200rpm by a magnetic stirrer. The stratum corneum was facing the donor compartment and the skin sample and formulation were placed between the two compartments (El Sayed et al., 2006).

#### *2.5.2 Skin preparation for permeation investigations*

Albino mice (20-25g) were bought from CAHF, Jamia Hamdard. The mice were treated with institutional standards for care. The mice were sacrificed. The skin of the abdomen was removed. An electric clipper was used to remove the dorsal hairs, and isopropyl alcohol (IPA) was applied to the excised skin to remove the subcutaneous fat. The skin was then thoroughly cleaned with distilled water before being physically checked for injury. The skin was stored at -21°C under an aluminium foil cover, and it was utilized within a week (El Sayed et al., 2006).

#### 2.5.3 Skin stabilization

The donor compartment was facing the stratum corneum of the skin, which was placed between both the two half cells after being shaved to the proper size. After being sonicated for 30 minutes to get rid of any dissolved gases, phosphate buffer (pH 7.4) was put into the receptor compartment. 1% sodium lauryl sulphate, and mechanically stirred at 200 rpm. The temperature in the diffusion cell was set to 37 °C ± 0.5 °C. To keep the skin stable, the buffer solution was changed every 30 minutes. When there were no apparent UV absorption bands after the skin had been stabilised for four to five hours, the skin was deemed to be stable (El Sayed et al., 2006).

#### *2.5.4 Permeation studies*

The fluids were poured into the receptor cell, and the donor compartment's skin surface was exposed to an ethosomal formulation containing Khellin and Fumaric acid. To ensure optimal mixing, the receptor media was magnetically stirred at 200 rpm and maintained at 37 ±0.5 °C. After adding the test formulation to the donor side,1 ml of an aliquot was taken from the receiver cell at predetermined intervals (i.e., initial, 30 mins,1, 2, 4, 6, 8, 10, 12 and 24 hr) for 24 hours and promptly replaced with the same volume of fresh media. Following the proper dilutions, the samples were filtered using a 0.45µm membrane filter, and a UV spectrophotometer was used to measure the amount of drug in the receptor media (El Sayed et al., 2006).

## 3. RESULT AND DISCUSSION

### 3.1 PRE-FORMULATION STUDIES

#### 3.1.1 Physical properties

The physical properties of the drug sample, including colour, odour, melting point, and others, were examined (Table No.2).

**Table 2:** Physical Properties of Drug(s)

| S. No. | Khellin | | | |
|---|---|---|---|---|
| | Analysis items | Specifications | Results | Test Methods |
| 1 | Identification | Positive | Confirms | TLC |
| 2 | Physical appearance | Crystalline powder | Confirms | Visual |
| 3 | Colour | Cream | Confirms | Visual |
| 4 | Odour | Characteristic | Confirms | organoleptic test |
| 5 | Melting point | 154-1550C | 153.66 $O_C$ | Capillary method |

### 3.1.2 Tests for identification

**a) UV analysis:**

The Khellin and Trans-butenedioic acid were dissolved in appropriate solvents and examined for their UV maxima (λmax), which are shown in Figure No. 1 and listed in the Table below.

| Drug | Solvent | UV maxima |
|---|---|---|
| Khellin | Methanol | 282nm |

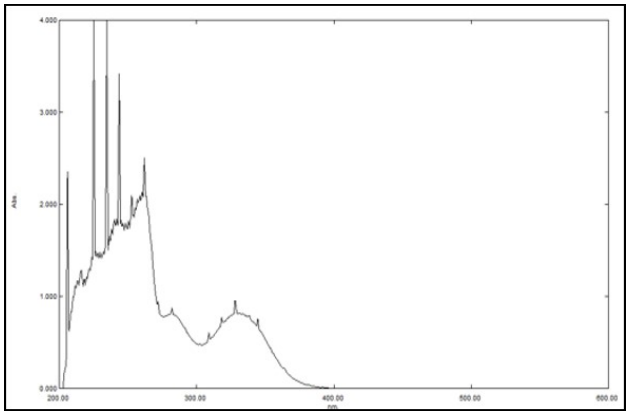

**Figure 1:** UV Spectrum of Khellin

### *3.1.3 IR Absorption spectrum of Khellin*

Khellin was present in the sample as indicated by its IR spectrum (Figure No. 2). The existence of an alkene group was indicated by absorption in the IR spectra at 1660.78 $cm^{-1}$, which is consistent with medium C=C stretching. Strong C=O stretching-related absorption at 1788.80 cm-1 indicated the existence of conjugated acid halide in the structure. These results collectively indicated that the IR spectrum matched Khellin's structural requirements.

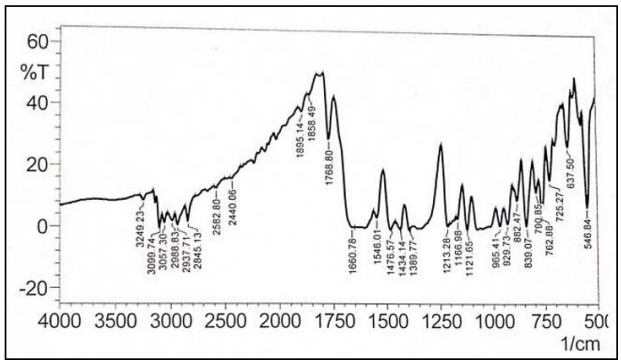


**Figure 2:** IR Spectra of Khellin

### 3.1.4 Differential Scanning Calorimetry (DSC)

Khellin was identified in the sample by the D.S.C. Analysis. The Khellin melting pointwas determined to be 153.669°C shown in Figure No.3 and Table No. 3.

**Table 3:** Melting points of Compound(s) determined through DSC

| | Melting point °C | |
|---|---|---|
| | Observed | Reported |
| Khellin | 153.669°C | 154 and 155 °C |

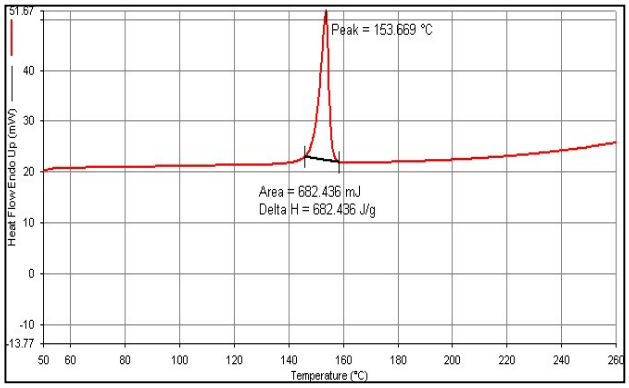


**Figure 3:** DSC thermograph of Khellin.

### 3.2 Analytical Methodology for Formulation

### *3.2.1 Determination of λmax by U.V. spectrum*

The λmax of Khellin was observed in different media as shown in Table No. 4.

**Table 4:** λmax observed in Methanol and PBS (pH 7.4)

| Compound | Methanol | PBS (pH 7.4) |
|---|---|---|
| **Khellin** | 282nm | 316nm |

### 3.2.2 Khellin calibration curves preparation

***a) In methanol***

The Khellin calibration curve was prepared in methanol at λmax of 282 nm for UV analysis. In the case of Khellin, the correlation coefficient ($R^2$ = 0.9959) shows a positive relationship between the two variables, concentration and absorbance. The Table No. 5, include a list of the concentrations and their related absorbances.

**Table 5:** Concentarion versus Absorption at 282nm (methanol)

| S. No. | Concentration (µg/ml) | Absorbance at 282 nm | Absorbance (Mean ± SD) |
|---|---|---|---|
| 1 | 2 | 0.216 | 0.215±0.001 |
| 2 | 4 | 0.234 | 0.233±0.001 |
| 3 | 8 | 0.264 | 0.264±0.001 |
| 4 | 16 | 0.287 | 0.286±0.001 |
| 5 | 20 | 0.312 | 0.312±0.001 |

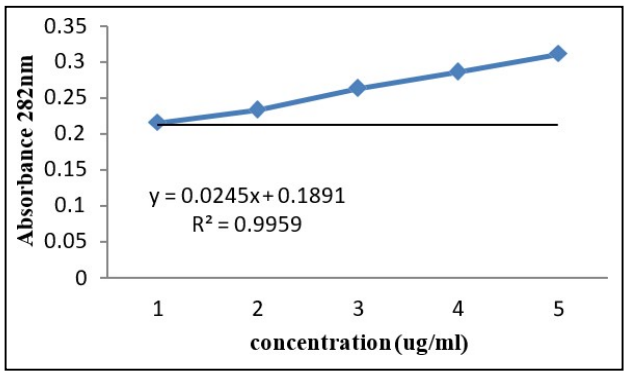


**Figure 4:** Calibration curve of Khellin in Methanol

***b) In PBS (pH 7.4)***

For UV analysis, the Khellin calibration curve was prepared in PBS (pH 7.4) at λmax of 316 nm. The correlation coefficient ($R^2$ = 0.9956) for Khellin shows a positive association between the two variables, concentration and absorbance. Concentration and the corresponding absorbance presented in Table No. 44 and Figure No. 56.

**Table 6:** Concentration versus absorbance at 316nm in PBS (pH 7.4)

| S. No. | Concentration (µg/ml) | Absorbance at 316 nm | Absorbance (Mean ± SD) |
|---|---|---|---|
| 1 | 2 | 0.162 | 0.161±0.001 |
| 2 | 4 | 0.209 | 0.208±0.001 |
| 3 | 8 | 0.273 | 0.272±0.001 |
| 4 | 16 | 0.318 | 0.317±0.001 |
| 5 | 20 | 0.387 | 0.386±0.001 |

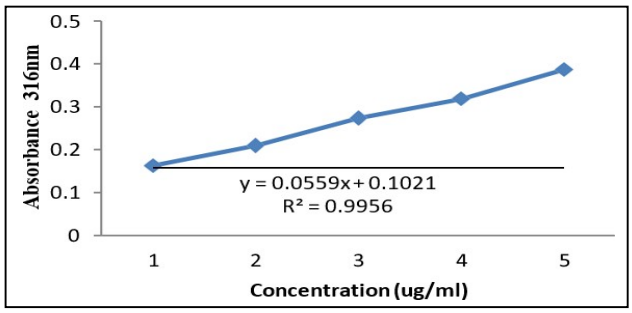


**Figure 5:** Calibration curve of Khellin in PBS (pH 7.4)

### 3.3 Formulation Development and Characterization

***3.3.1 KHL loaded ethosomal formulation optimization:***

A design expert software was utilised to develop the formulation. This Table No. 47, includes the experimental designs and results that BBD provided. When the data from the produced formulations was entered into the Design Expert software, a polynomial quadratic model was recommended with a p-value of <0.0001 for each of the three components. Additionally, all three variables predicted R-Square values were reasonably consistent with their adjusted R-Square values (Table No. 48).

**Table 7:** KHL Formulations provided by BBD (RSM Design Expert Software)

| Code | Independent values | | | Dependent values | | |
|---|---|---|---|---|---|---|
| | A: Phopholipid (mg) (A) | B: Ethanol (%) (B) | C: Sonication time (mins) (C) | Vesicle size(nm) (R 1) | PdI (R 2) | EE (%) (R 3) |
| KHL 1 | 15 | 85 | 2 | 217.5 | 0.429 | 69.3 |
| KHL 2 | 10 | 80 | 4 | 272.5 | 0.487 | 45.3 |
| KHL 3 | 10 | 80 | 2 | 264.7 | 0.468 | 79.3 |
| KHL 4 | 20 | 80 | 4 | 301.3 | 0.547 | 82.9 |
| KHL 5 | 15 | 80 | 3 | 211.2 | 0.382 | 61.2 |
| KHL 6 | 15 | 85 | 4 | 241.6 | 0.439 | 46.6 |
| KHL 7 | 15 | 80 | 3 | 210.6 | 0.374 | 61.8 |
| KHL 8 | 15 | 80 | 3 | 210.7 | 0.375 | 62.7 |
| KHL 9 | 10 | 75 | 3 | 252.8 | 0.452 | 48.5 |
| KHL10 | 15 | 80 | 3 | 210.5 | 0.376 | 61.7 |
| KHL11 | 15 | 75 | 2 | 233.5 | 0.392 | 63.5 |
| KHL12 | 20 | 80 | 2 | 293.7 | 0.536 | 91.9 |
| KHL13 | 20 | 85 | 3 | 282.8 | 0.545 | 75.7 |
| KHL14 | 10 | 85 | 3 | 242.4 | 0.439 | 65.4 |
| KHL15 | 20 | 75 | 3 | 269.2 | 0.481 | 84.2 |
| KHL16 | 15 | 75 | 4 | 223.7 | 0.433 | 44.3 |
| KHL17 | 15 | 80 | 3 | 210.5 | 0.375 | 60.8 |

**Table 8:** Regression Analysis (Khellin)

| Model (Quadratic) | Response $Y_1$ | Response $Y_2$ | Responses $Y_3$ |
|---|---|---|---|
| R2 | 0.9999 | 0.9981 | 0.9986 |
| Adjusted $R^2$ | 0.9999 | 0.9957 | 0.9969 |
| Predicted $R^2$ | 0.9995 | 0.9802 | 0.9873 |
| SD | 0.3438 | 0.0040 | 0.7946 |
| %CV | 0.1409 | 0.8968 | 1.22 |
| $y_1$=+210.70+14.32A+0.6375B+3.71C+6.00AB-0.0500AC+8.47BC+52.54$A^2$-1.44 $B^2$+1 9.81 $C^2$.....*(Equation No.a)* | | | |
| $y_2$=+0.3764+0.0329A+0.0118B+0.0101C+0.0192AB-0.0020AC-0.0077BC+0.0946$A^2$+0.0083$B^2$+0.0386 $C^2$....*( Equation No.b)* | | | |
| $y_3$= +61.64+12.03A+2.06B-10.61C-6.35AB+6.25AC-0.8750BC+12.87 $C^2$.....*(Equation No.c)* | | | |

** $Y_1$ – vesicle size, $Y_2$- PdI and $Y_3$ -Entrapment efficiency*

### 3.3.2 Effect of A, B, and C on vesicle size

Independent factors impact on dependent variables along with their interactive effects is presented in equations No. (a), (b) and (c). Equation No.a reveals the individual interactive effect of the amount of phospholipid (A), ethanol % (B) and sonication time (C) on Y.

According to this equation, A, B and C exerted a positive effect on particle size. A combination of variables like AB, AC and BC positively influenced particle size. This can also be understood from 3D response graphs.

KHL 10 exhibited a particle size of 210.5nm while KHL 4 exhibited a particle size of 301.3nm.

In general, it was found that when phospholipid concentration increased from 15-20 mg, particle size increased. However, when 20mg phospholipid concentration was used, maximum particle sizes were observed. This might be due to the interactive effects of the amount of Ethanol concentration and sonication time.

### *3.3.3 Effect of A, B, and C on PdI*

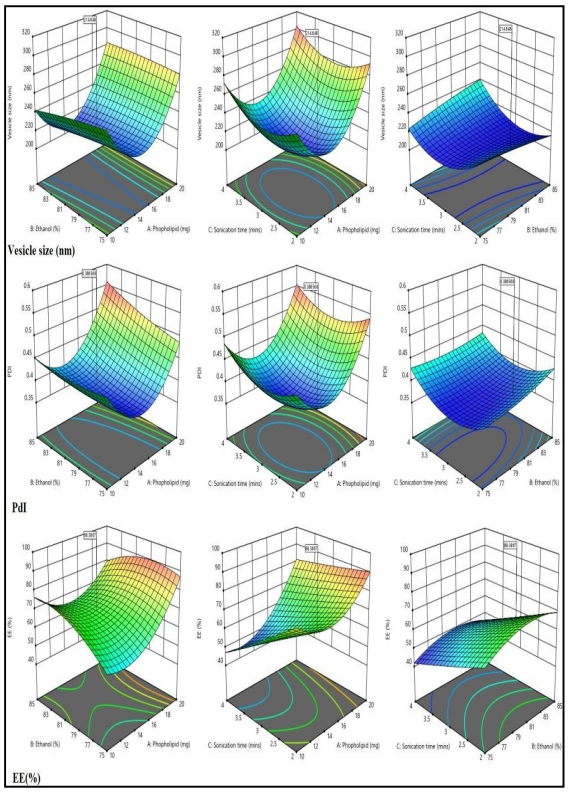


***Figure 6:*** *Three-dimensional (3D) response surface graphs showing relationshipbetween (Phospholipid, Ethanol and sonication time) with responses (Vesicle size, PDI, entrapment efficiency)*

KHL-7 showed the lowest PdI (0.374) while KHL-4 showed a maximum PdI of 0.547.In general, it was seen that the amount of ethanol percentage and sonication time positively impact PdI. It was also seen that a greater quantity of lipids indulged greaterPdI. Equation No. (b) shows the individual interactive impact of A, B and C on $Y^2$. A and B had a negative influence on PdI. Combinational interactive effects AC and BC showed mixed effects on $Y_2$.

### *3.3.4 Effect of A, B, and C on EE*

KHL-12 exhibited an entrapment efficiency of 91.9% while KHL-16 demonstrated an entrapment efficiency of 44.3%. The phospholipid in greater quantities caused a reduction in entrapment efficiencies. Equation No. c show how variables A, B and C effects entrapment efficiencies Y3. As can be seen from the equation, AC and BC influenced the response positively. However, AB displayed a detrimental effect on Y3.

### *3.3.5 Validation of Optimised conditions for KHL formulation*

To "minimize" particle size, "minimize" PdI, and "maximize" entrapment efficiency, constraints have been applied to the dependent variables. The solutions obtained were Phopholipid-14.50mg, Ethanol-80.19(%) and Sonication time-2.35mins. Responses obtained were Particle size- 214.64nm, a PDI -0.387 and entrapment efficiency- 68.38%. Check trails were done with similar optimized conditions (Phospholipid- 15mg, Ethanol- 80% and Sonication time-2 mins as furnished by BBD.

The responses obtained were- Particle size-218.73, a PdI-0.394 and entrapment efficiency-71.97%

### 3.6 Characterization of KHL Loaded Ethosomal Formulation(s)

**Size distribution analysis for vesicles**

According to Malvern Instruments measurements, the ethosome vesicles average size falls between 200 and 300 nm, which will be further considered. All of the ethosomes had a narrow size distribution, according to the Polydispersity index (PdI).

**Table 9:** Particle size and PdI of Khellin loaded ethosomal formulation

| Formulation code | Z-Average (d.nm) | PdI |
|---|---|---|
| KHL | 220.9 | 0.369 |

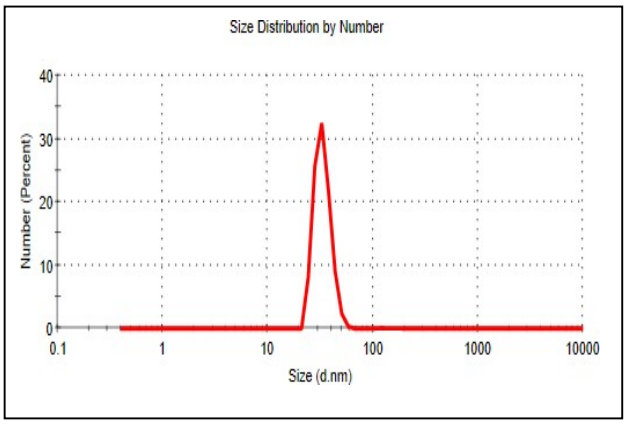


**Figure 7:** Particle size distribution of KHL Formulation

### *3.3.6 Vesicular shape & Surface morphology*

The optimized formulation, TEM microphotograph ultimately revealed that the vesicles were spherical and evenly dispersed across the nano-range of vesicles. The SEM microphotograph showed that the surface of vesicles was in conformation.

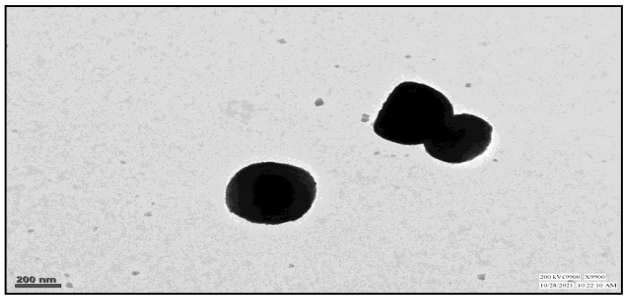


**Figure 8:** TEM microphotograph of KHL formulation

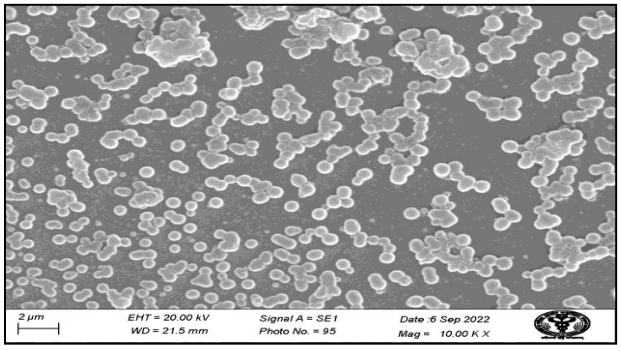


**Figure 9:** SEM microphotograph of KHL formulation

### *3.3.7 Fourier Transform Infrared Absorption Spectrum*

The and developed formulation FTIR spectra (Figure No.63) revealed bands at 1660.78 cm-1(medium C=C stretching), and 1788.80 (Strong C=O stretching) respectively. After interpretation of the spectra, it was concluded that there was no interaction between the drug Khellin and the excipients used in the formulation of ethosomes.

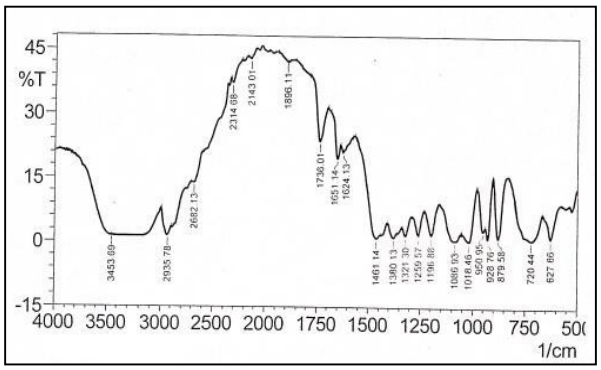


**Figure 8:** IR Spectra of KHL loaded formulation

### 3.3.8 DSC Analysis (Differential Scanning Colorimetry)

The developed drug-loaded ethosomal formulation DSC analysis was performed. To observe any interactions between the drug and excipients used in the formulation. DSC thermogram of the sample is given below (Figure No.64).

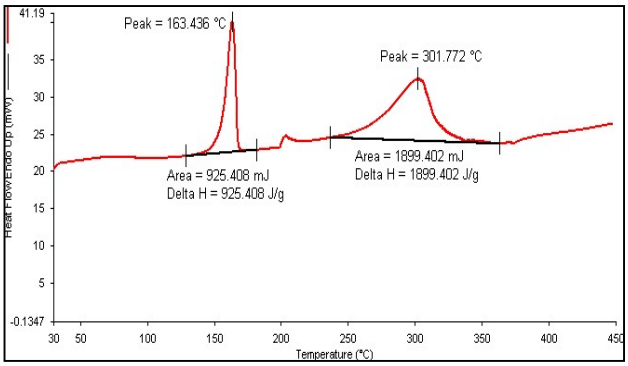


**Figure 81:** DSC thermogram of KHL loaded formulation

## CONCLUSION

The findings of this study indicate that khelin-loaded ethosomes offer a promising approach for improved topical drug delivery. By encapsulating khelin in ethosomes, we achieved enhanced skin penetration and retention, which is essential for effective dermal drug delivery. The ethosomal formulation showed high encapsulation efficiency, nanoscale particle size, and stability under refrigerated conditions, suggesting its robustness as a drug carrier. In vitro release studies demonstrated sustained release, highlighting the

ethosomal vesicles' potential to control khelin release over time. Additionally, the skin permeation studies confirmed a marked improvement in khelin's permeation through the stratum corneum compared to traditional formulations. This novel approach, leveraging the ethosomal carrier system, can potentially enhance the bioavailability and efficacy of khelin in treating dermatological conditions. Further studies on clinical efficacy and safety can provide more insights, paving the way for possible commercial applications of khelin-loaded ethosomes in skin therapeutics.

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