RESEARCH PAPER

Development and Characterization of Benincasa Hispida Extract-Loaded Nanocochleates for Enhanced Drug Delivery

Ms. Aaditi S. Punekar[1*], Dr. Abhijeet D. Kulkarni[2]

[1,2] School of Pharmaceutical Sciences, Sandip University, Nashik, Maharashtra, India

[*] **Corresponding Author:** Ms. Aaditi S. Punekar. Email: aaditipunekar789@gmail.com

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ABSTRACT

Nanocarriers in herbal medicine delivery have garnered considerable interest for their capacity to improve the bioavailability, stability, and therapeutic efficiency of plant-derived bioactive chemicals. Benincasa hispida, sometimes referred to as winter melon or ash gourd, is a medicinal plant widely used in traditional medicine for its many pharmacological attributes. The clinical use of Benincasa hispida extract is constrained by its inadequate water solubility, fast degradation, and diminished bioavailability, which impede its absorption and therapeutic efficacy. Lipid-based nanocarriers, including nanoliposomal and nanocochleates, have developed as effective delivery strategies to address these problems. We have developed and analyzed Nanocochleates consisting of cholesterol and soya lecithin in molar ratios as an effective oral nanocarrier for delivery applications. The batches were created using a Response Surface Methodology (RSM) called Box-Behnken design, using Design Expert® software (Version 13.0). The Box-Behnken design had three independent variables: Cholesterol amount (mg) (A), Soya lecithin (mg) (B), and hydration volume (C). The examined dependent variables were particle size (nm), polydispersity index (PDI), and zeta potential (mV). The Box-Behnken design included factorial points, a central point, and axial points, resulting in 13 experimental runs. The produced nanoliposomes were analyzed using Drug Content, Entrapment Efficiency, Particle Size, Zeta Potential, In Vitro Franz Diffusion Study, FTIR Spectroscopy, and Transmission Electron Microscopy. The HF9 batch exhibited exceptional performance. Thereafter, extract-loaded nanocochleates were manufactured using the trapping method. The resulting liposomes were vortexed, and 100 microliters of a 0.1 M calcium chloride solution was gradually introduced. This quickly led to the extract liposomal phase becoming murky, indicating the formation of nanocochleates. The nanocochleates were further evaluated for drug content, encapsulation efficiency, zeta potential, and particle size. The optimized batch NF1 exhibited advantageous results.

KEYWORDS: Benincasa hispida, Nanoliposomes, Nanocochleates.

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1. INTRODUCTION:

The use of nanocarriers in herbal drug delivery has gained significant attention due to their potential to enhance the bioavailability, stability, and therapeutic efficacy of plant-derived bioactive compounds [1-3]. Benincasa hispida, commonly known as winter melon or ash gourd, is a medicinal plant extensively used in traditional medicine for its diverse pharmacological properties, including antioxidant, anti-inflammatory, antimicrobial, and neuroprotective effects. However, the clinical application of Benincasa hispida extract is limited by its poor aqueous solubility, rapid degradation, and low bioavailability, which hinder its absorption and therapeutic potential. To overcome these challenges, lipid-based nanocarriers such as nanoliposomal and nanocochleates have emerged as promising delivery systems. [4-10]

Nano liposomes are bilayer vesicular systems composed of phospholipids that encapsulate hydrophilic and lipophilic molecules, thereby improving drug stability and controlled release. They provide an effective means of delivering plant extracts by enhancing their solubility, protecting them from enzymatic degradation, and facilitating cellular uptake. However, nanoliposomes are relatively unstable and prone to fusion and leakage, which can affect drug

retention and bioavailability[11-14]

To address these limitations, nanoliposomes can be converted into nanocochleates through the are solid, spiral-shaped lipid carriers that offer superior structural stability, increased drug protection, and enhanced intracellular uptake via membrane fusion and endocytosis. These properties make nanocochleates an ideal platform for the sustained and targeted delivery of herbal bio actives. [15-19]

The development of Benincasa hispida extract-loaded nanoliposomes and nanocochleates represents a novel approach to optimizing the pharmacokinetics and pharmacodynamics of its bioactive constituents. The preparation of nanoliposomal formulations involves techniques such as thin-film hydration, ethanol injection, and sonication, which facilitate the encapsulation of plant extracts within lipid bilayers. The subsequent conversion of nanoliposomes into nanocochleates through calcium ion-mediated aggregation enhances their physicochemical stability and bioavailability. These nanoformulations have the potential to improve drug solubility, provide sustained release, enhance permeability, and target-specific drug delivery, making them valuable for pharmaceutical and nutraceutical applications [20-21]. This study focuses on the formulation and characterization of Benincasa hispida extract-loaded nanoliposomes and nanocochleates to evaluate their potential as an advanced drug delivery system.

2. MATERIAL AND METHODS:

Materials

The fresh fruit of *Benincasa hispida* was collected from Wagh Nursery, Pune –Solapur Road. Acetone, ethanol, cholesterol, soya lecithin, and chloroform were procured from Solanki Enterprise, Pune**,** and were of analytical grade,

Methods

Method of Extraction [22]

Whole fruit was (*Benicasa hispida*) washed and whole fruit was cut into small cubes and grind it. Make three parts of the grinded fruit pulp, and maceration was done with three solvents Ethyl Acetate, Ethanol, Water. Keep for 24 hrs and filtered with muslin cloth and stored in refrigerator. 00 grams of pulp is first prepared and taken in a suitable extraction container. To this, 450 ml of the selected solvent (water, ethanol, or ethyl acetate) is added separately for each trial. The mixture is then thoroughly mixed and allowed to undergo extraction for a specific period, with occasional stirring or shaking to ensure proper contact between the solvent and the pulp. After the extraction incorporation of divalent cations such as calcium of magnesium. Nanocochleates

process is complete, the mixture is filtered to separate the liquid extract from the solid residue. The collected filtrate is then measured to determine the total amount of extract obtained. The same procedure is repeated for each solvent under identical conditions to maintain consistency.

Quantitative Phytochemical Analysis [23-25]:

For flavonoid determination, 100 µl of sample extract in methanol is mixed with 100 µl of 20% aluminum trichloride, a drop of acetic acid is added, and the mixture is diluted to 5 ml with methanol. After standing for 40 minutes, absorbance is measured at 415 nm against a blank, with quercetin used as the standard. For terpenoid estimation, 1 g of extract is macerated with ethanol and filtered; 2.5 ml of filtrate is then mixed with phosphomolybdic acid and concentrated sulfuric acid, allowed to stand for 30 minutes, diluted to 12 ml with ethanol, and absorbance is recorded at 700 nm.

Thin Layer Chromatography [26-27]:

Thin layer chromatography is carried out to identify phytochemicals using specific solvent systems. Flavonoids are separated using toluene, ethyl acetate, glacial acetic acid, and water as the mobile phase, followed by visualization under UV light at 365 nm and spraying with anisaldehyde-sulfuric acid reagent. Terpenoids and carotenoids are separated using cyclohexane and ethyl acetate as the mobile phase and observed under UV light at 268 nm for detection.

Method of liposomes preparation [28]

Extract-loaded liposomes were prepared using the thin film hydration method to form multilamellar vesicles (MLVs). In this procedure, a lipid phase consisting of cholesterol and soya lecithin in specific molar ratios was prepared, and 100 mg of the extract was dissolved along with the lipids in a mixture of chloroform and ethanol (1:1 v/v, 5 ml each). The resulting solution was then evaporated under vacuum at 45°C using a rotary flash evaporator, leading to the formation of a thin, dry lipid film on the inner wall of the flask. This lipid film was subsequently hydrated with phosphate buffer (pH 7.4) and vigorously agitated using a vortex mixer to facilitate vesicle formation after complete removal of residual solvents. Finally, the formed liposomes were subjected to sonication to reduce their size and obtain small unilamellar vesicles.

Optimization of Liposome Preparation:

A thin, uniform coating is crucial for determining the final product of the liposomal preparation. During the hydration and film formation processes, the rotational speed was maintained between 60 and 100 rpm.

Formulation and optimization using Box Behnken design

In this study, a Response Surface Methodology (RSM) known as Box Behnken design was utilized with Design Expert® software (Version 13.0). The Box Behnken design involved three independent variables: the amount of Cholesterol (mg)) (A), Soya lecithin (mg) (B), and the hydration volume (C). The dependent variables examined were Particle size (nm), PDI and Zeta potential (mv). The Box Behnken design included factorial points, a center point and axial points, resulting in a total of 13 experimental runs

Evaluation and characterization of Liposomes:

Measure 1 ml of Nanoliposomal formulation using a pipette and transfer it into a 10 ml volumetric flask. Dilute to 10 ml with methanol to achieve the desired concentration for UV analysis. Filter the diluted solution through Whatman filter paper No. 1 or a suitable membrane filter to remove any particulate matter. Measure the absorbance of the filtered solution at the drug's specific wavelength at 280 nm using a UV spectrophotometer (Jasco V-630).

$$Drug\ content(\%) = \frac{\text{Amount of drug in the formulation}}{\text{Amount of drug theoretically added}} \times 100 \quad \dots (1)$$

Drug Content [29]

continuous stirring at 100 rpm, and 2 ml of the nanoliposomal formulation was placed in the donor compartment. At predetermined time intervals (1, 2, 3, 4, 5, and 6 hours), 5 ml samples were withdrawn from the receptor compartment and immediately replaced with an equal volume of fresh buffer. The collected samples were appropriately diluted and analyzed using a UV-Vis spectrophotometer at 252 nm (λmax) to determine drug concentration. The drug release mechanism from the nanoliposomal formulation was further analyzed by fitting the in vitro diffusion data to various kinetic models to understand the release behavior.

Entrapment Efficiency [30]

Liposomal formulation was taken in a volumetric flask and diluted up to 10 ml with Methanol. The diluted formulation was taken in Eppendorf tubes and centrifuged at 10,000 rpm for 2 h at 4 °C. The supernatant was collected carefully and filtered. The filtered sample was analyzed by UV (Jasco Uv-630) at 280 nm to get the drug present in supernatant.

$$\boldsymbol{Entrapment\ efficiency}(\%) = \frac{\boldsymbol{Added\ drug - Free\ drug}}{\boldsymbol{Added}} \times \mathbf{100} \quad \mathbf{\dots (2)}$$

Particle size and zeta potential [31]

The 3-5ml nanoliposomal formulation was taken and mixed with distilled water and sonication was kept for 30 min. The analysis was performed at a temperature of 25 ℃. Same procedure repeated at zeta potential.

In vitro Franz diffusion study [32]

he in vitro drug release of the liposomal formulation was evaluated using a vertical Franz diffusion cell with phosphate buffer solution (PBS) at pH 6.5 as the receptor medium. A freshly prepared phosphate buffer of pH 6.5 was used, and the Cellophane membrane (molecular weight 12,000, pore size 2.4 nm) was soaked in the receptor medium overnight before the experiment. The diffusion system was maintained at 37 °C ± 2 °C on a multistation diffusion apparatus with

FTIR Spectroscopy [33]

The drug excipients compatibility study was performed by FTIR technique. The Optimized batch (HF9) samples were scanned over wave number range of 500-4000 cm-1 with diffraction reflectance scanning technique.

Transmission Electron Microscopy [34]

TEM is done for analysis of surface morphology. Transmission Electron Microscopy (TEM) The surface morphology of the optimized batch HF9 was established by using Transmission Electron Microscopy (TEM).

Few microliters of diluted Nanocochelate solution of optimized batch HF9 was put on 300 mesh copper grid film coated with copper and were air-dried at room temperature. Once it was dried completely, the sample was stained using a 2 %w/v phosphotungstic acid solution removing the excess with a filter paper. Further, the analysis and images of samples were captured by using digital micrograph and Soft Imaging Viewer Software.

Preparation of Nanocochleates from Optimized Liposomal Batch (HF9) [28]

Extract-loaded nanocochleates were prepared using the trapping technique. The resulting liposomes were vortexed, and 100 microliters of a 0.1 M calcium chloride solution was added drop by drop. This

immediately caused the extract liposomal phase to become turbid, indicating the formation of nanocochleates. These nanocochleates were then evaluated for drug content, encapsulation efficiency, zeta potential, and particle size.

Evaluation of Nanocochelates

Drug Content [29]

Measure 1 ml of Nanocochelate formulation using a pipette and transfer it into a 10 ml volumetric flask. Dilute to 10 ml with methanol to achieve the desired concentration for UV analysis. Filter the diluted solution through Whatman filter paper No. 1 or a suitable membrane filter to remove any particulate matter. Measure the absorbance of the filtered solution at the drug's specific wavelength at 252 nm using a UV spectrophotometer (Jasco V-630).

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In vitro Franz diffusion study [32]

The in vitro drug release of the nanocochleate formulation was evaluated using a vertical Franz diffusion cell (DBK Sr No-210796) with phosphate buffer solution (PBS) at pH 6.5 as the receptor medium. A freshly prepared phosphate buffer of pH 6.5 was used, and the Cellophane membrane (molecular weight 12,000, pore size 2.4 nm) was soaked in the receptor medium overnight before the study. The diffusion assembly was maintained at 37 °C ± 2 °C on a multistation diffusion apparatus with continuous stirring at 100 rpm, and 5 ml of the formulation was placed in the donor compartment. At predetermined time intervals (1, 2, 3, 5, and 6 hours), 2 ml samples were withdrawn from the receptor compartment and immediately replaced with an equal volume of fresh buffer. The collected samples were suitably diluted and analyzed using a UV-Vis spectrophotometer at 252 nm (λmax) to determine drug concentration. The mechanism of drug release from the nanocochleate formulation was further assessed by fitting the obtained data to various kinetic models to understand the release behavior.

Transmission Electron Microscopy [34]

TEM is done for analysis of surface morphology. Transmission Electron Microscopy (TEM) The surface morphology of the optimized batch NF1 was established by using Transmission Electron Microscopy (TEM).

Few microliters of diluted Nanocochelate solution of optimized batch NF1 was put on 300 mesh copper grid film coated with copper and were air-dried at room temperature. Once it was dried completely, the sample was stained using a 2 %w/v phosphotungstic acid solution removing the excess with a filter paper. Further, the analysis and images of samples were captured by using digital micrograph and Soft Imaging Viewer Software.

3. RESULTS AND DISCUSSION:

Quantitative Phytochemical Analysis:

The preliminary phytochemical analysis of the test samples revealed that both plant extracts contained various bioactive compounds, with positive results for multiple phytochemicals. To further understand their composition, quantitative phytochemical analysis was performed to determine the concentration of specific compounds in each extract. The analysis focused on key phytochemicals such as flavonoids and terpenoids, and the results were supported by TLC verification. Among the extracts, the ethanol extract demonstrated the highest content, with flavonoids measured at 9.21 ± 0.030 mg/gm and terpenoids at 4.23 ± 0.042 mg/gm, indicating superior extraction efficiency. The ethyl acetate extract showed moderate levels, with flavonoids at 1.03 ± 0.072 mg/gm and terpenoids at 1.63 ± 0.092 mg/gm. In contrast, the aqueous (water) extract had the lowest concentrations, with flavonoids at 0.29 ± 0.011 mg/gm and terpenoids at 0.54 ± 0.020 mg/gm, suggesting that water was less effective in extracting these phytochemicals compared to ethanol and ethyl acetate.

Thin layer chromatography of bioactive factions:

The thin layer chromatography analysis was carried out to identify the chemical constituents present in the ethanol extract using specific mobile The preliminary phytochemical analysis of all test sample evaluated. Both plants extract exhibited positive results for different phytochemicals. In continuation of phytochemical analysis quantitative estimation assistance to determine their concentration or proportion in individual extract. In Quantitative phytochemical analysis all test extracts observed details of results for selected phytochemicals reported in table. Phytochemical compounds such as flavonoids, and terpenoids were screened in the extract by quantitative means. Ethanol extract showed more proportion of defined components verified by TLC analysis. The quantitative phytochemical analysis revealed variations in flavonoid and terpenoid content spraying with anisaldehyde–sulfuric acid reagent followed by observation under UV light at 365 nm. The Rf value of the test ethanol extract was found to be 0.61, which is close to the standard reference value of 0.63, indicating the presence of flavonoids. Similarly, terpenoids and carotenoids were analyzed among different extracts. The ethanol extract showed the highest concentration of both flavonoids (9.21 ± 0.030 mg/gm) and terpenoids (4.23 ± 0.042 mg/gm), indicating its superior extraction efficiency for these compounds. The ethyl acetate extract exhibited moderate levels, with flavonoids at 1.03 ± 0.072 mg/gm and terpenoids at 1.63 ± 0.092 mg/gm. In contrast, the aqueous (water) extract showed the lowest content, with flavonoids measured at 0.29 ± 0.011 mg/gm and terpenoids at 0.54 ± 0.020 mg/gm, suggesting comparatively lower effectiveness in extracting these phytochemicals effectiveness in extracting these phytochemicalsphases and visualization techniques. Flavonoids were separated using a mobile phase of toluene, ethyl acetate, glacial acetic acid, and water (100:11:11:26), and were visualized by

using a mobile phase of cyclohexane and ethyl acetate (75:25) and visualized under UV light at 268 nm. The observed Rf value for the test extract was 0.52, which closely matched the standard value of 0.54, confirming the presence of terpenoids and carotenoid

Evaluation and characterization of liposomes

Table 2. Evaluation and Characterization of HF1 to HF13

Formulation Batches	Drug content (%)	Entrapment Efficiency (%)	Particle Size	PDI
HF1	75.00±0.12	73.15±0.14	246.2	0.323
HF2	84.05±0.31	71.81±	396.4	0.219
HF3	88.18±0.56	70.97±	259.9	0.321
HF4	86.06±0.01	71.68±	242.4	0.414
HF5	85.49±0.48	72.93±	275.4	0.312
HF6	79.38±0.33	73.61±	298.1	0.426
HF7	68.82±0.24	67.07±	289.7	0.245
HF8	91.09±0.14	78.17±	426.6	0.342
HF9	91.21±0.01	87.20±	201.1	0.212
HF10	91.16±0.78	83.98±	213.8	0.216
HF11	90.49±0.95	80.88±	279.3	0.274
HF12	83.18±0.34	78.64±	277.3	0.298
HF13	84.18±0.12	78.84±	281.9	0.301

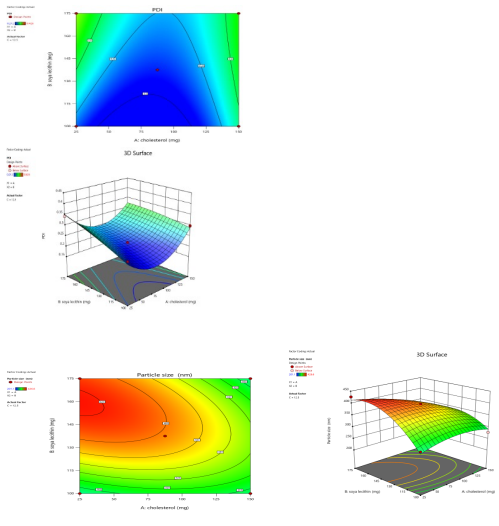

Figure 1. Counter Plot and 3D Surface of Particle Size and PDI

In vitro Franz diffusion study

The in vitro drug release study for all formulations (HF1–HF13) showed a clear time-dependent release pattern, with drug release gradually increasing over the 6-hour period. Initial release at 1 hour varied among formulations, reflecting differences in composition and drug diffusion characteristics. Among all batches, HF9 demonstrated the highest and most consistent cumulative release, indicating it as the optimized formulation. Overall, the data show that all formulations provided sustained, progressive drug release, with HF9 exhibiting superior release performance, making it the most efficient and promising batch for enhanced drug delivery.

FTIR Spectroscopy

The FTIR analysis of formulation HF9 confirmed the presence of key functional groups, indicating successful formulation. The O–H stretch of carboxylic acids was observed at 2980.45 cm^{-1} and 2843.52 cm^{-1}, which falls within the reported range of 3300–2500 cm^{-1}, though slightly shifted, possibly due to hydrogen bonding or interactions with other components. The O–H stretch of alcohols or phenols appeared at 3348.78 cm^{-1}, aligning well with the expected range of 3500–3200 cm^{-1}, confirming the presence of hydroxyl groups. The N–H bending vibration at 1635.34 cm^{-1} was within the reported range of 1650–1580 cm^{-1}, indicating the presence of amine functional groups. Additionally, the C–C aromatic ring stretch was detected at 1455.99 cm^{-1}, within the expected range of 1500–1400 cm^{-1}, confirming aromatic ring structures. The C–O stretch of alcohols appeared at 1012.45 cm^{-1}, 1053.91 cm^{-1}, and 1032.69 cm^{-1}, aligning well with the reported range of 1320–1000 cm^{-1}, indicating the presence of ether or alcohol functionalities. Overall, the FTIR spectrum of HF9 suggests the successful incorporation of functional groups relevant to the formulation,

Transmission Electron Microscopy

The TEM analysis of the optimized batch HF9 reveals a polydisperse distribution of nanoparticles, with sizes ranging from approximately 22.5 nm to 194.2 nm. The majority of the particles appear to be in the nanometer range, with a few larger aggregates present. The morphology of the nanoparticles is predominantly spherical, which is beneficial for stability and controlled drug release. However, some degree of aggregation is visible, suggesting possible particle interactions or the need for further stabilization. The size variation indicates a moderate level of uniformity, though the presence of larger particles may slightly impact formulation consistency. Overall, the nanosized particles (<200 nm) suggest potential advantages in drug delivery, such as enhanced bioavailability and sustained release, though further refinement may be needed to optimize Dispersity and minimize aggregation.

Evaluation and chracterization of nanocochleates:

Drug Content and Entrapment Efficiency

The evaluation of different formulations showed variations in drug content and entrapment efficiency. Formulation NF1 exhibited a higher drug content of 91.18±0.12% along with an entrapment efficiency of 87.16±0.02%, indicating better incorporation of the drug within the vesicles. In comparison, formulation NF2 showed a slightly lower drug content of 84.59±0.36% and an entrapment efficiency of 81.77±0.14%, suggesting comparatively reduced performance in drug loading and retention. Overall, NF1 demonstrated superior formulation characteristics compared to NF2.

Particle size and zeta potential

The characterization of the formulations revealed differences in particle size, polydispersity index (PDI), and zeta potential. Formulation NF1 exhibited a smaller particle size of 102.3 nm with a lower PDI of 0.178, indicating a more uniform size distribution, along with a zeta potential of -27.5 mV, suggesting better stability. In contrast, formulation NF2 showed a larger particle size of 125.3 nm and a higher PDI of 0.265, indicating comparatively less uniformity, with a zeta potential of -24.1 mV, reflecting slightly lower stability than NF1. Overall, NF1 demonstrated more favorable physicochemical characteristics compared to NF2.

In vitro Franz diffusion study

The in vitro drug release study showed a gradual increase in drug release for both formulations over time. At the initial time (0 hr), no drug release was observed. After 1 hour, NF1 showed a release of 9.0±0.14%, while NF2 exhibited 5.0±0.65%. At 2 hours, the release increased to 17.5±0.65% for NF1 and 15.4±0.45% for NF2. Interestingly, at 3 hours, NF2 (23.3±0.98%) showed slightly higher release than NF1 (20.3±0.01%). From 4 hours onward, NF1 again demonstrated comparatively higher release, with 29.2±0.87% at 4 hours and 42.6±0.36% at 5 hours, while NF2 showed 27.7±0.02% and 41.9±0.37% respectively. By 6 hours, NF1 achieved a significantly higher cumulative release of 91.4±0.01% compared to 84.7±0.24% for NF2, indicating overall better drug release performance.

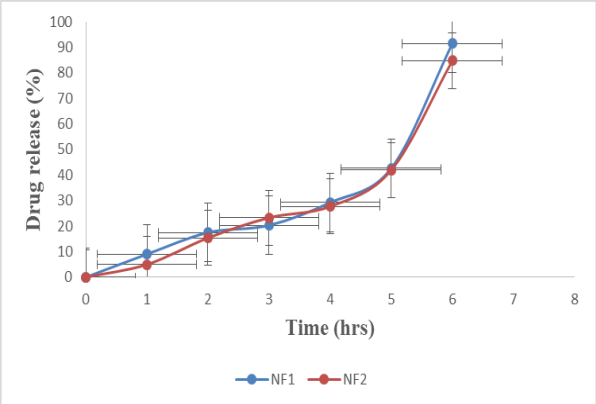

Figure 2. Drug release of NF1-NF2

Fourier transform infra-red spectroscopy

The Fourier-transform infrared (FTIR) analysis of the sample revealed characteristic functional groups. An O–H stretching vibration was observed at 3336.25 cm^{-1}, which falls within the reported range of 3200–3500 cm^{-1}, indicating the presence of hydroxyl groups. Additionally, an N–H bending vibration was detected at 1635.34 cm^{-1}, consistent with the reported range of 1580–1650 cm^{-1}, confirming the presence of amine or amide groups in the sample.

Transmission Electron Microscopy

The TEM image of the optimized batch NF1 reveals a well-dispersed nanoparticle system with spherical morphology. The particle size distribution ranges from 6.5 nm to 98.4 nm, indicating the presence of uniformly distributed nanoparticles. The majority of the nanoparticles fall within the nano-range (<100 nm), which is ideal for enhanced drug penetration and bioavailability. The smaller particle sizes (e.g., 6.5 nm, 18.4 nm, 34.7 nm) suggest a high surface area, which may contribute to improved drug release and absorption. Additionally, the lack of significant aggregation indicates good formulation stability.

4. CONCLUSION:

We have developed and investigated Nanocochleates comprised mixtures of cholesterol and soya lecithin in molar ratios as an effective oral nanocarrier for the delivery. The batches were formulated using, a Response Surface Methodology (RSM) known as Box Behnken design by utilizing Design Expert® software (Version 13.0). The Box Behnken design involved three independent variables: the amount of Cholesterol (mg)) (A), Soya lecithin (mg) (B), and the hydration volume (C). The dependent variables examined were Particle size (nm), PDI and Zeta potential (mv). The Box Behnken design included factorial points, a center point, and axial points, resulting in a total of 13 experimental runs. The formulated nanoliposomes were characterized using Drug Content, Entrapment Efficiency, Particle size and zeta potential , In vitro Franz diffusion study , FTIR Spectroscopy and Transmission Electron Microscopy. It was observed that the batch HF9 showed excellent results. After wards, Extract-loaded nanocochleates were prepared using the trapping technique. The resulting liposomes were vortexed, and 100 microliters of a 0.1 M calcium chloride solution was added drop by drop. This immediately caused the extract liposomal phase to become turbid, indicating the formation of nanocochleates. These nanocochleates were then evaluated for drug content, encapsulation efficiency, zeta potential, and particle size. The optimized batch NF1 exhibited good results.

5. CONFLICT OF INTEREST:

The authors declare that they have no conflicts of interest concerning this article.

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