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Original Article

Fabrication and Characterization of *Azadirachta indica* oil Induced Nanoemulgel Using 3^3 Central Composite Design (CCD): Assessment of Antibacterial Activity

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ABSTRACT

Background: In this study, *neem* oil's therapeutic potential against bacterial inflammations is explored through nanoemulsion technology. Despite its historical significance in traditional medicine, *neem* oil formulations face challenges such as limited stability and poor solubility. Nanoemulsions offer a promising solution by enhancing *neem* oil's efficacy, stability, and patient acceptability. Utilizing Response Surface Methodology (RSM), specifically Central Composite Design (CCD), enables systematic optimization of nanoemulsion formulations, enhancing their therapeutic potential. **Materials and Methods:** In this research, the Central Composite Design was instrumental in fine-tuning parameters like the concentration of surfactant (A), speed of homogenization (B), and running time of homogenization (C). These variables were explored across three different levels. Particle size, serving as the dependent variable, was assessed as the response to variations in these independent factors. Mathematical equations and response surface plots were used to understand the relation between the factors influencing the outcome and the resulting dependent variable. **Results:** The optimized CCD model has a particle size of 97.9 nm, a zeta potential of -21.0 mV, and a PDI value of 0.512. Carbopol 934 was utilized in formulating the nanoemulgel. The observed responses closely resembled the anticipated outcomes from the optimized process. Morphological analysis and *in vitro* release studies were employed to characterize the prepared nanoemulgel formulation. **Conclusion:** The Response Surface Methodology facilitates the formulation of *neem* oil emulsion with the smallest droplet size possible. Furthermore, the nanoemulgel exhibited significant antibacterial activity against *Staphylococcus aureus* (*S. aureus*).

Keywords: *Azadirachta indica* (*Neem* Oil), Central Composite Design (CCD), Response Surface Methodology (RSM), Nanoemulgel.

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INTRODUCTION

Neem (*Azadirachta indica*) oil is well-known for its antimicrobial properties and has a long history of utilization in traditional medicine, notably within the Indian Ayurvedic system.[1] Its effectiveness against a wide range of bacterial strains, including both Gram-positive and Gram-negative types, highlights its broad-spectrum antibacterial activity, making it valuable for addressing various bacterial infections.[2] While traditional *neem* oil formulations for topical drug delivery have certain drawbacks such as limited stability, poor solubility, and reduced bioavailability, nanoemulgel formulations offer solutions to overcome these challenges and provide enhanced efficacy, stability, patient acceptability, and reduce the risk of side effects.[3]

Nanoemulsion formulation involves various factors such as oil phase composition, surfactant concentration, and processing parameters (e.g., homogenization time, and speed). Response Surface Methodology (RSM) allows for the systematic exploration of these factors and their interactions in a relatively small number of experiments, leading to efficient optimization compared to traditional One-Factor-At-a-Time (OFAT) methods.[4] Through the application of statistical methodologies like Central Composite Design (CCD) or Box-Behnken Design (BBD), RSM efficiently explores the design space and identifies the optimal formulation conditions. In this research, we applied CCD for the formulation of the *neem* oil nanoemulsion.[5] This enables us to develop mathematical models that describe the behavior of the system, facilitating prediction and optimization of nanoemulgel

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formulation. In this research, Gram-positive bacteria were employed to evaluate the antibacterial activity of the optimized nanoemulgel formulation.[6]

MATERIALS AND METHODS

Neem oil obtained from a local store in Guntur served as the dispersed oil phase. Tween 80, a non-ionic surfactant, was procured from Loba Chemie Pvt., Ltd. in Mumbai. Deionized water provided by Thermo Fisher Scientific India Pvt., Ltd. in Mumbai was utilized for the aqueous phase. Cabopol 934, acquired from Loba Chemie Pvt., Ltd., was employed for the gel base, while Triethanolamine and Propylene glycol, obtained from Thermo Fisher Scientific India Pvt., Ltd. and Loba Chemie Pvt., Ltd. respectively, were also utilized in the formulation.

Screening of Surfactants for Nanoemulsion

After conducting rigorous screening encompassing phase behavior analysis, emulsification efficiency (including factors such as emulsion stability, droplet size, and appearance), and compatibility testing,[7] a range of surfactants such as Tween 80, Tween 20, Span 80, Span 20, and PEG 400 were assessed. Following a thorough evaluation, Tween 80 emerged as the optimal surfactant for the nanoemulsion formulation due to its exceptional stability and superior solubility with the *neem* oil.

Preparation of *Neem* oil Nanoemulsion

The nanoemulsion was fabricated using the high-speed homogenization technique. The oil phase contained *neem* oil and Polysorbate 80 (Tween 80), whereas the aqueous phase consisted of deionized water. The two phases were meticulously combined by slowly adding the aqueous phase drop by drop into the oil phase while maintaining constant stirring using a magnetic stirrer.[8] After an initial stirring duration of 30 min, the mixture underwent additional processing via high-speed homogenization for another 30 min.

Optimization of Nanoemulsion by Design of Experiments (DoE)

Experimental design

To fabricate the *neem* oil nanoemulsion, a Central Composite Design with 16 experimental runs, employing a 3-factor and 3-level approach was developed using JMP Statistical Software (version 17.0.0, Student subscription).[9] This design facilitates the examination of quadratic response surfaces and the development of second-order polynomial models. The CCD includes factorial points, a central point, and axial points, which define the limits of the experimental region.[10] Through this design, The primary effects, interactions, and quadratic influences of the formulation ingredients can be extensively explored and optimized within a predetermined area of interest. The design matrix comprised 16 experimental runs, incorporating 2 central points to enhance the robustness and reliability of the optimization process, as depicted in Table 1.

The software will produce nonlinear quadratic equations representing the design. These equations encapsulate the relationships between the factors and responses in the experimental runs.

Whereas Y=Response, N_0=The intercept denotes the arithmetic mean of all 16 experimental runs. N_1 to N_9=The regression coefficient corresponds to the observed experimental values of the response variable Y. X_1, X_2, and X_3=Independent variable with code levels, X_1 X_2 and X_3=Linear interaction terms, X_1 2, X_2 2 and X_3 2=Quadratic terms.

In this design matrix, the independent variables were the concentration of surfactant Tween 80 (X1), homogenization time (X2), and homogenization speed (X3). The variables were represented by coded values of +1 and -1, indicating high and low values, respectively, as illustrated in Table 2. The dependent variable, Particle size (Y1), was the response measured after preparing the 16 formulations, and it was fitted into the design to analyze the effects of the independent variables. Prediction Profiler is used to identify optimal factor settings that either maximize or minimize the response variable, depending on experimental goals.

Heating Cooling Cycle

The refined formulation underwent characterization to assess its thermodynamic stability through a cooling-heating cycle. The formulation was initially stored at 4°C for 48 hr, followed by incubation at 48°C for another 48 hr. The process of heating and cooling was reiterated three times.[11] Subsequently, samples of the nanoemulsions were inspected for any signs of phase separation or precipitation.

Freeze-thaw Cycle

The optimized nanoemulsion underwent freeze-thaw cycling, involving freezing it at -20°C for 24 hr, followed by thawing at room temperature.[12] This sequence was repeated three times. Afterward, the formulation underwent centrifugation analysis to assess any potential phase separation.

Stability Index

The stability index of the optimized essential oil Nanoemulsion (NE) was assessed following three consecutive freeze-thaw cycles, employing the subsequent equation.[13]

Macroscopic Stability

To evaluate the stability of the prepared formulation, a centrifugation method was employed. The optimized emulsion formulation underwent a centrifugation study to assess its kinetic stability. The nanoemulsion was subjected to centrifugation at varying speeds, including 1000, 2000, and 3000 RPM, each for a

duration of 15 min. Subsequently, the macroscopic stability of the formulation was evaluated by contrasting the appearance of the emulsion before and after the centrifugation process.[14]

Morphology study using Scanning Electron Microscope (SEM)

Dilute the nanoemulsion to an appropriate concentration to avoid excessive aggregation or overlapping of droplets on the substrate. Use a suitable solvent or dispersant (water) for dilution. Choose a suitable substrate (silicon wafers) for mounting your nanoemulsion sample. Make sure the substrate is pristine and devoid of any contaminants that might disrupt the SEM analysis. Apply a small volume of the diluted nanoemulsion onto the selected substrate. Ensure uniform spreading of the nanoemulsion across the substrate surface.[15] Use a pipette or a similar tool to control the amount of nanoemulsion applied. Allow the nanoemulsion sample to dry completely to fix the droplets onto the substrate surface. The drying process can be achieved by air-drying at room temperature. Mount the dried nanoemulsion sample onto an SEM stub or holder using conductive carbon tape or adhesive. Ensure that the specimen is firmly affixed to the stub to prevent any movement or displacement while being imaged. Transfer the mounted sample into the SEM chamber and ensure that the instrument is properly calibrated for imaging. Use appropriate SEM settings such as accelerating voltage, beam current, and magnification for imaging the nanoemulsion sample. Capture SEM images at various magnifications to analyze the surface morphology of the nanoemulsion droplets.

Preparation of Nanoemulgel

A Carbopol gel was formulated by adding 0.5% w/v of Carbopol 934 into deionized water. The specified quantity of Carbopol was dispersed in deionized water and mixed using a mechanical stirrer for 20 min. Subsequently, 0.1% v/v of triethanolamine and 10% v/v of propylene glycol were added dropwise and stirred for an additional 10 min to form a suitable gel base for the formulation.[16] The nanoemulsion formulation was added to the gel base to produce the nanoemulgel using a mechanical stirrer.

Characterization of nanoemulgel

The formulations' color is assessed against both black and white backgrounds. The odor of the gels is evaluated by blending a small

Table 1: Coded values of 16 runs by CCD.

Runs	Tween 80 (X_1)	Time in min (X_2)	RPM (X_3)
1	+	+	+
2	a	0	0
3	0	0	A
4	-	-	+
5	0	0	a
6	0	a	0
7	+	-	+
8	-	+	+
9	A	0	0
10	-	-	-
11	0	A	0
12	0	0	0
13	-	+	-
14	+	+	-
15	0	0	0
16	+	-	-

Table 2: Variables and their levels in CCD.

Factor	Independent (I) & Dependent (D) variables	Low (-)	High (+)
I.1	Amount of surfactant (ml) X1	4	5
I.2	Homogenization time (min) X2	25	30
I.3	Homogenization speed (RPM) X3	10000	12000
D.1	Particle size (nm) (Y)	Minimize	Minimize

quantity of gel in water and then smelling it. The consistency of the formulations is examined by applying the gel to the skin. Visual inspection is conducted on all formulations to detect any aggregates after the gels have been set in their containers. Greasiness is evaluated by applying the formulation to the skin. Visual inspection is used to observe any phase separation.[17]

pH assessment

The pH of the emulgel was measured using a digital pH meter. The formulation was transferred into a beaker, and the pH meter was inserted into the formulation to take the measurement.[18] This procedure was repeated three times using the identical formulation, and the average of the recorded values was computed to ascertain the pH.

Viscosity determination

The gel's viscosity was assessed utilizing a Brookfield viscometer.[19] where measurements were taken at 10 RPM for a duration of 3 min using spindle 52.

Extrudability

The extrudability test was conducted using a hardness tester. 5 g of nanoemulsion gel were filled into aluminum collapsible tubes, ensuring proper placement of the plunger to secure the tube. A pressure of 1 g per square centimeter was applied for 30 sec.[20] The amount of nanoemulsion gel extruded from the tube was measured, and the procedure was repeated three times to ensure accuracy.

Swelling index

To determine the swelling index of the prepared nanoemulgel, 1 g of the formulation was positioned on porous aluminum foil and immersed separately in 50 mL beakers filled with 10 mL of 0.1 N NaOH solution. Samples were extracted from the beakers at different intervals and permitted to dry before undergoing reweighing.[21] The swelling index was determined using the following calculation method.

Where (SW) %=represents the equilibrium percent swelling, Wt=denotes the weight of the swollen emulgel after time t, and Wo=signifies the original weight of the emulgel at zero time.

Spreadability

The spreadability of the formulated nanoemulgel was assessed 72 hr after formulation by measuring the spread diameter between two glass plates after 1 min. A 400 mg sample of the nanoemulgel was weighed and deposited within a premarked 1 cm circle on a glass plate. Subsequently, a second plate was placed over it, and as pressure was applied to the upper plate, the diameter of the gel spread was observed.[22] The spreadability was determined using the subsequent equation.

Whereas, S=Spreadability of nanoemulgel, m=Weight placed on an upper glass plate (gm), 1=Length of the upper glass plate (cm), and T=time taken (Sec).

In vitro Antibacterial Studies

Preparation of Agar Plates

The process of preparing agar plates involved dissolving agar in water, followed by autoclaving the mixture at 121°C for 15 min. After autoclaving, the agar solution was cooled to a temperature range of 40-45°C. Subsequently, 25 mL of the liquefied agar was poured into each petri dish. The plates were then left to solidify under a laminar airflow hood.[23]

Preparation of Inoculum

Staphylococcus aureus (*S. aureus*) was selected as the inoculum for assessing the antibacterial effectiveness of topical formulations containing *Neem* oil Nanoemulgel. The microorganisms were subcultured in advance to ensure they were in the logarithmic phase of growth, thereby maintaining the accuracy and reliability of the experimental outcomes.[24]

Inoculation of Agar Plates

After the agar plates had solidified, the inoculum was applied to by streaking method. Following inoculation, the plates were left to air dry for approximately 5 min at ambient room temperature.[25]

Preparation of Agar well diffusion assay

The agar well diffusion assay was conducted using the previously dried plates. Wells were created in the inoculated agar plates using a sterile cork borer, with each well having a diameter of 5 mm. A precise quantity of the nanoemulgel formulation was added to each well of the plates. The plates were then placed in an incubator at 37°C for 72 hr, during which inhibition zones were observed.[26] The diameter of the inhibition zones was measured using a ruler, with precision to the nearest millimeter.

RESULTS

Optimization of Essential oil Nanoemulsion

A Central Composite Design was employed to optimize the essential oil Nanoemulsion, as demonstrated in Table 3. The impact of the independent factors on the dependent variables (response) was examined through a 3D response surface plot depicted in Figure 1. It was observed that increasing the speed of homogenization leads to the smaller size of nanoemulsion and simultaneously concentration of surfactant and running time of the homogenization also affect the particle size as shown in the 3D surface plots generated by JMP statistical software. Run 3 shows the 104.3 NM for the optimized nanoemulsion compared to the other runs at different levels, it was observed to be the least size. Moreover, the Prediction Profiler effectively improves processes by examining the influence of factors on a response

Table 3: Responses of runs generated by CCD methodology.

Runs	DoE Pattern	Tween 80 (X_1)	Time in min (X_2)	RPM (X_3)	Particle Size (Y)
1	+++	5	30	12000	112.1
2	a00	4	27.5	11000	106.7
3	00A	4.5	27.5	12000	104.3
4	−−+	4	25	12000	145.9
5	00a	4.5	27.5	10000	144.7
6	0a0	4.5	25	11000	140.3
7	+−+	5	25	12000	104.9
8	−++	4	30	12000	120.1
9	A00	5	27.5	11000	135.2
10	−−−	4	25	10000	113.2
11	0A0	4.5	30	11000	119.9
12	0	4.5	27.5	11000	138.0
13	−+−	4	30	10000	160.3
14	++−	5	30	10000	137.2
15	0	4.5	27.5	11000	138.7
16	+−−	5	25	10000	179.9

variable and forecasting outcomes using both experimental data and the model that has been fitted with the desirability of 0.994 and particle size in the range of 95.27 nm as shown in Figure 2.

Analysis of the response

The Analysis of Variance (ANOVA) outcomes reveals that the quadratic model for the response variable Y holds statistical significance. With an *F*-value of 3.04 exceeding the critical threshold of 2.5, it indicates that the model is significant, and thus, the null hypothesis can be rejected. Moreover, the *p*-value of 0.0159 indicates that the model terms, such as A, B, C, AB, AC, BC, A2, B2, and C2, are all statistically significant ($p<0.05$). This implies that surfactant concentration, homogenization time, and speed are the independent variables that significantly influence the response variable Y. Furthermore, the lack-of-fit value indicates that the P-value is greater than the significant level, indicating that there is inadequate evidence to refute the null hypothesis. This suggests that the model adequately fits the data.

The quadratic equation for the response Y was provided in terms of coded factors.

Y=130.97+2.25 N1- 3.38 N2-14.76 N3- 7.04 N1 N2-11.56 N1 N3-2.88 N2 N3-3.70 N1 2+5.59 N2 2-0.20 N3 2.

The equation illustrates the quantitative impacts of all three independent variables N1, N2, and N3 as the primary factors influencing the response Y. Additionally, the interaction terms N1N2, N1N3, and N2N3 indicate non-linear relationships between the response Y and variables when changed simultaneously. The interaction profiler determines whether two or more factors have a great influence on the response. For response Y, factors N1, and N2 both show shallow slopes with sight curvature. It indicates that the concentration of surfactant and homogenization time were most important as represented in Figure 3.

The response surface plots illustrate the correlation between the independent variables and the dependent variable. Analysis of the interaction between factors N1, N2, and N3 was conducted regarding the response of particle size. It was observed from the response plot that there exists an interaction between the concentration of surfactant, homogenization time, and homogenization speed, impacting the particle size of the Nanoemulsion.

The Pareto chart arranges sorted data in descending order, displaying bars that represent individual values and a line indicating the cumulative total. This combination of bar and line graphs aids in identifying the most significant categories by visually comparing the heights of the bars. The line plot provides insight into the cumulative impact as we progress through the list of categories. Commonly utilized in quality control, process improvement, and decision-making contexts, the Pareto chart helps prioritize efforts and resources based on the most influential factors contributing to a problem or outcome. In our data analysis as per Figure 4, factors N3, N1N3, and N1N2 emerge as the most significant categories influencing the Nanoemulsion.

Evaluation of Nanoemulsions

Heating - cooling cycle

After the centrifugation test, the optimized nanoemulsion underwent a series of heating-cooling cycles to evaluate its thermodynamic stability. It was observed that after three consecutive heating-cooling cycles. There was no evidence of creaming or phase separation in the nanoemulsion. This observation confirms that the nanoemulsion exhibited thermodynamic stability.

Freeze-Thaw cycle

After subjecting the optimized nanoemulsion to freeze-thaw cycles, it was noted that no phase separation occurred, even after undergoing three successive freeze-thaw cycles. However, there was an increase in particle size from 97.9 nm to 110.4 nm, and the Polydispersity Index (PDI) also increased from 0.512 to 0.612.

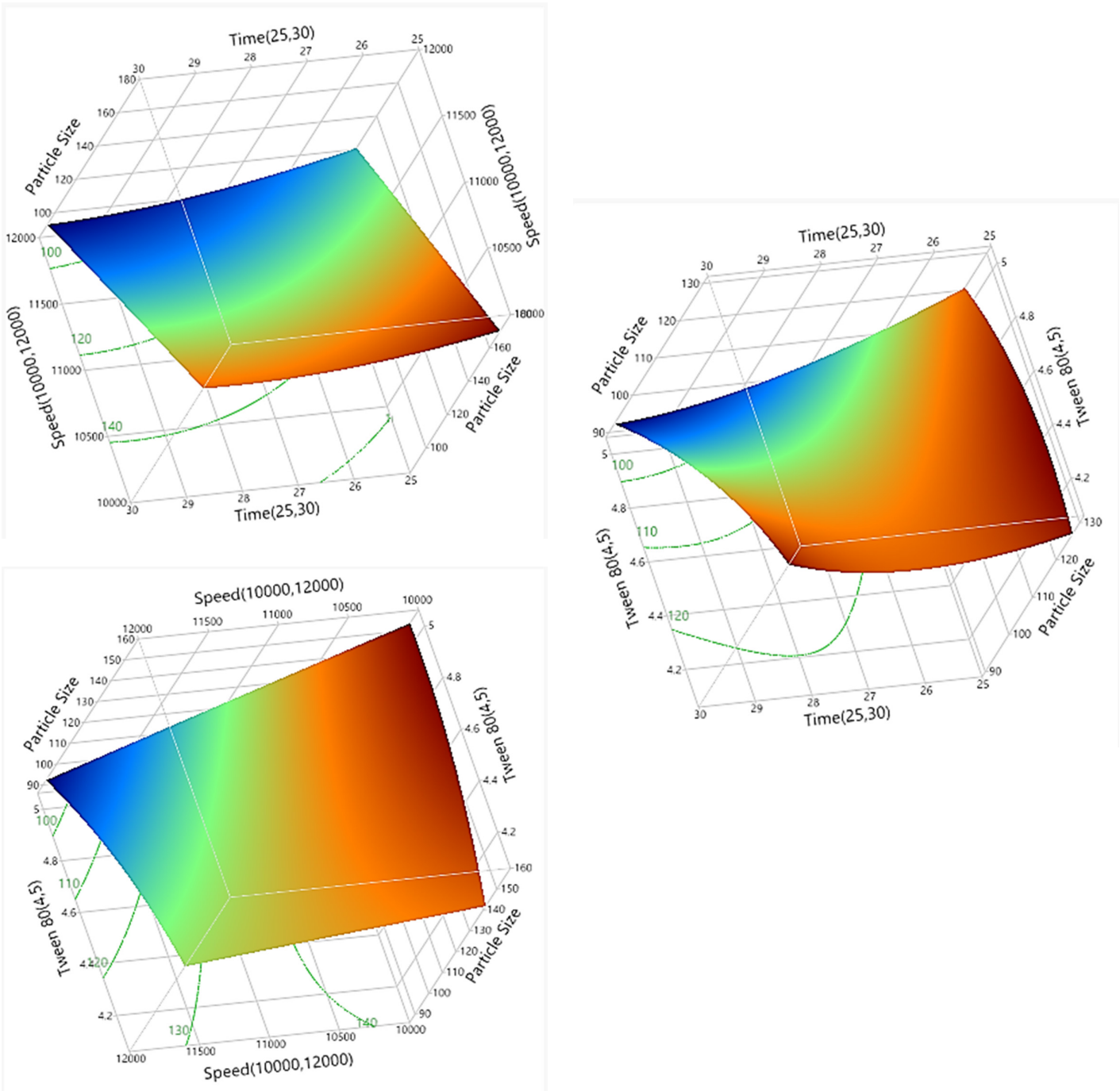

Figure 1: 3D graph showing the effect of independent factors on response particle size (Y). (A) effect of speed and time on the particle size (B) effect of surfactant and speed on particle size (C) surfactant and time on particle size.

Table 4: **kinetic stability of nanoemulsion.**

Stirring speed*	Phase separation	creaming	Flocculation
1000	Not detected	Not detected	Not detected
2000	Not detected	Not detected	Not detected
3000	Not detected	Not detected	Not detected

* revolutions per minute.

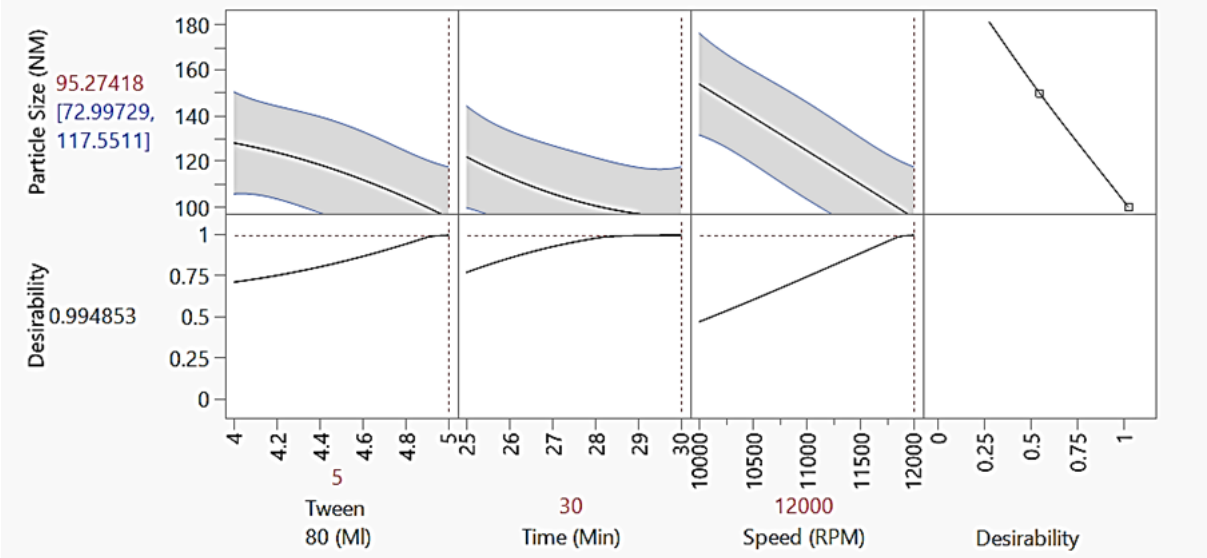

Figure 2: Prediction Profiler of Nanoemulsion.

Figure 3: Interaction Profile of optimized Nanoemulsion.

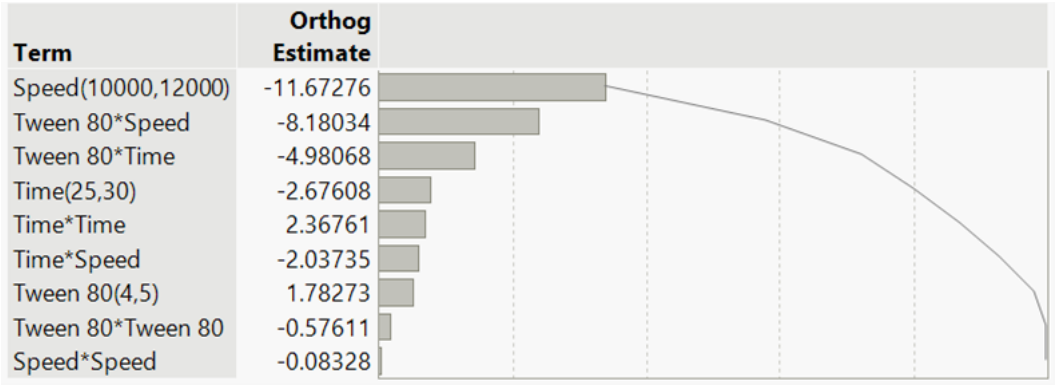

Figure 4: Pareto Plot of optimized Nanoemulsion.

Stability index of nanoemulsions

The stability index of the nanoemulsion formulation was found to be 87.23%. This index serves as a quantitative measure indicating the extent to which the formulation maintains its structural integrity and uniformity over time. A stability index of 87.23% suggests a high level of robustness and resilience in maintaining the desired properties of the nanoemulsion.

Macroscopic stability

The optimized nanoemulsion formulation underwent centrifugation analysis to assess its kinetic stability. It was subjected to centrifugation at speeds of 1000, 2000, and 3000 rpm for 15 min each. Despite applying different centrifugal forces, there was no evidence of phase separation, suggesting that the formulation maintained stability under these conditions, as indicated by the results presented in Table 4.

ATR-FTIR Analysis

The compatibility between the active ingredients and excipients in the nanoformulations was investigated through Fourier Transform Infrared (FTIR) analysis using the Attenuated Total Reflectance (ATR) method. This analytical approach allows for a detailed examination of chemical interactions and molecular structures present within the formulations. Spectral scanning was conducted on samples including neem oil, neem oil nanoemulsion, and neem oil nanoemulgel formulations, covering the infrared range from 4000 to 400 cm^{-1}, thereby generating IR spectra. Figure 5 illustrates the obtained IR spectra, providing a visual representation of the molecular vibrational frequencies and characteristic peaks present in each sample. By comparing the spectra of the individual components (*neem* oil) with those of the formulated nanoemulsion and nanoemulgel, any shifts, peaks, or changes in intensity can be identified. These changes indicate potential interactions or modifications occurring between the active ingredients and excipients during the formulation process.

SEM Analysis

SEM analysis was utilized to investigate the surface morphology of the Nanoemulsion formulation, enabling the observation of globules in three dimensions. Samples were scrutinized at different magnifications and an ideal accelerating voltage, usually set at 5 kV. It provides a thorough examination of the surface morphology of the dispersed phase within the formulation. Automated image analysis tools were employed to effectively analyze the shape and surface morphology, as depicted in Figure 6.

Evaluation characteristics of nanoemulgel

The evaluation of the optimized nanoemulgel formulation involves a comprehensive analysis of various characteristics to ensure its efficacy, safety, and suitability for its intended application. Tables 5 and 6 outline the assessment criteria for the different tests conducted, providing a structured framework for observation and analysis. These criteria encompass a range of parameters, including physical appearance, spreadability, viscosity, pH, swelling index, consistency, and phase separation among others.

In vitro antimicrobial studies

The *in vitro* antibacterial assessment entailed comparing the diameter of inhibition zones (mm) between the nanoemulgel and gel base formulations. The gel base served as the control, with a predetermined quantity used for comparison. Results revealed significant antibacterial effectiveness of the *Neem* oil nanoemulgel against *S. aureus*, as illustrated in Figure 7. Notably, the nanoemulgel formulation displayed a substantial inhibition zone measuring 6±1.2 cm, surpassing the smaller inhibition zone of the gel base. This underscores the potent antimicrobial properties of the *neem* oil nanoemulgel against *S. aureus*, aligning with previous literature findings.

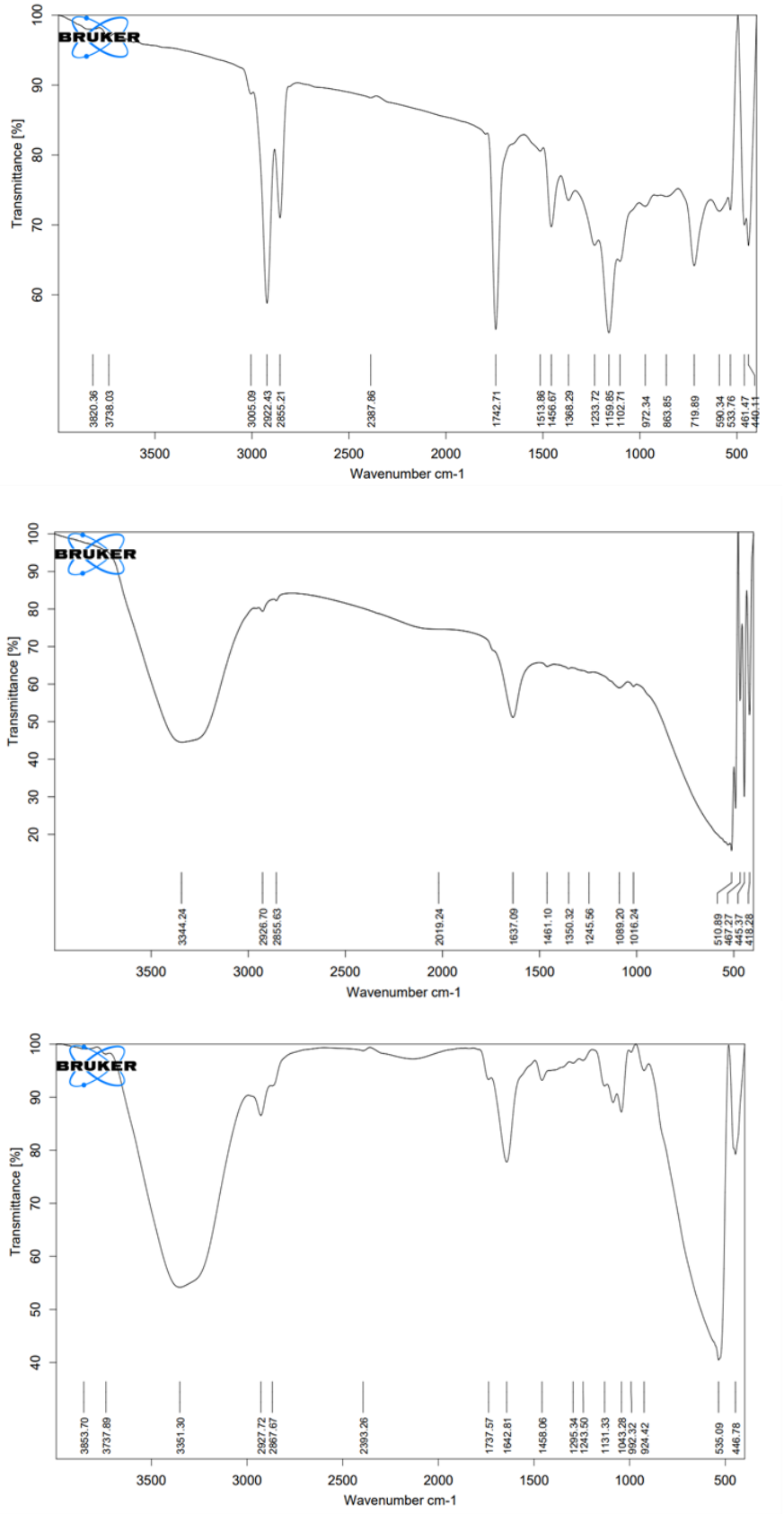

Figure 5: (A) ATR FTIR of *Neem* Oil (B) ATR FTIR of *Neem* oil Nanoemulsion (C) ATR FTIR of *Neem* Oil Nanoemulgel formulation.

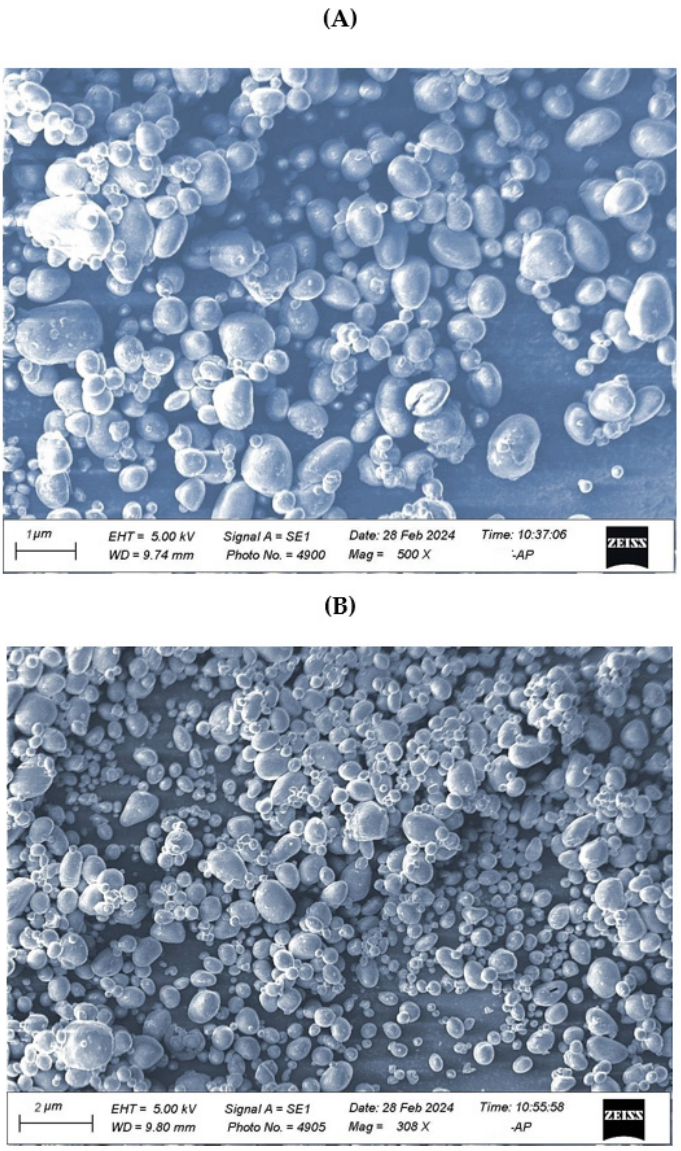

Figure 6: The SEM Images of the Nanoemulsion formulation (A) SEM under 1 micrometre region (B) SEM under 2 micrometre regions.

Table 5: Physical characteristics of Nanoemulgel formulation.

Evaluation parameters	Results
color	Light yellowish
Odor	Sulphuric, nutty aromas
consistency	Smooth texture and ease of spreadability
Homogeneity	Uniform distribution and stable emulsion
Greasiness	Less greasiness
Phase separation	None

Table 6: Evaluation parameters of nanoemulgel formulation

Evaluation parameters	Results
pH	6.78±0.21
Viscosity (cps)	344.1±2
Extrudability	0.76±0.41
Swelling index	17±0.15
spreadability	9.1±0.2

*(n=3).

DISCUSSION

ATR-FTIR analysis was conducted on *Neem* oil, *neem* oil-loaded Nanoemulsion, and Nanoemulgel formulations. The results indicated that the active ingredient peaks had reduced intensities in the Nanoemulsion formulation, verifying the encapsulation of the essential oil within the Nanoemulsion.[27] The Nanoemulsion exhibited a particle size of 106.7 nm in run 2, which was the smallest among the 16 runs in the CCD design space. This particle size is directly influenced by factors such as homogenization speed, homogenization time, and surfactant concentration. A minimal concentration of emulsifier in nanoemulsions can potentially result in the fusion of smaller particles generated during homogenization, thereby increasing particle size.

Furthermore, an increase in the dispersed phase also contributes to larger particle sizes in the nanoemulsion.[28] The duration of homogenization (X2) was identified as the second factor affecting the particle size (Y) of the nanoemulsion. It was observed that the duration of homogenization had a notable impact on the particle size of the nanoemulsion. Furthermore, factor 3, representing homogenization speed, exhibited a notable impact on the size of the nanoemulsion particles. Elevating the speed from 10,000 rpm to 12,000 rpm resulted in discernible alterations in the particle size (Y) of the Nanoemulsion.

The optimized formulation displayed a zeta potential of -21.0 mV. It was noted that as the particle size of the nanoemulsion decreased, the zeta potential of the formulations increased. This phenomenon can be attributed to the fact that a reduction in particle size enhances the surface area, leading to an augmentation in surface charge. Zeta potential, an analytical method employed to gauge the surface charge of nanoparticles in colloidal dispersions, reflects these alterations in surface charge.[29] Nanoemulsions are kinetically stable. Therefore, the stability of freshly prepared nanoemulsion was assessed by subjecting it to stress conditions (centrifugation) to expedite emulsion breakage. In nanoemulsions, the free energy of the colloidal dispersion surpasses that of the separate phases, indicating their thermodynamic instability. However, they can be rendered kinetically stable by creating a significant energy barrier between the phases. Following three consecutive freeze-thaw cycles, the formulation remained stable.

Nonetheless, there was a slight increase in particle size from 97.9 nm to 110.4 nm, and the Polydispersity Index (PDI) rose from

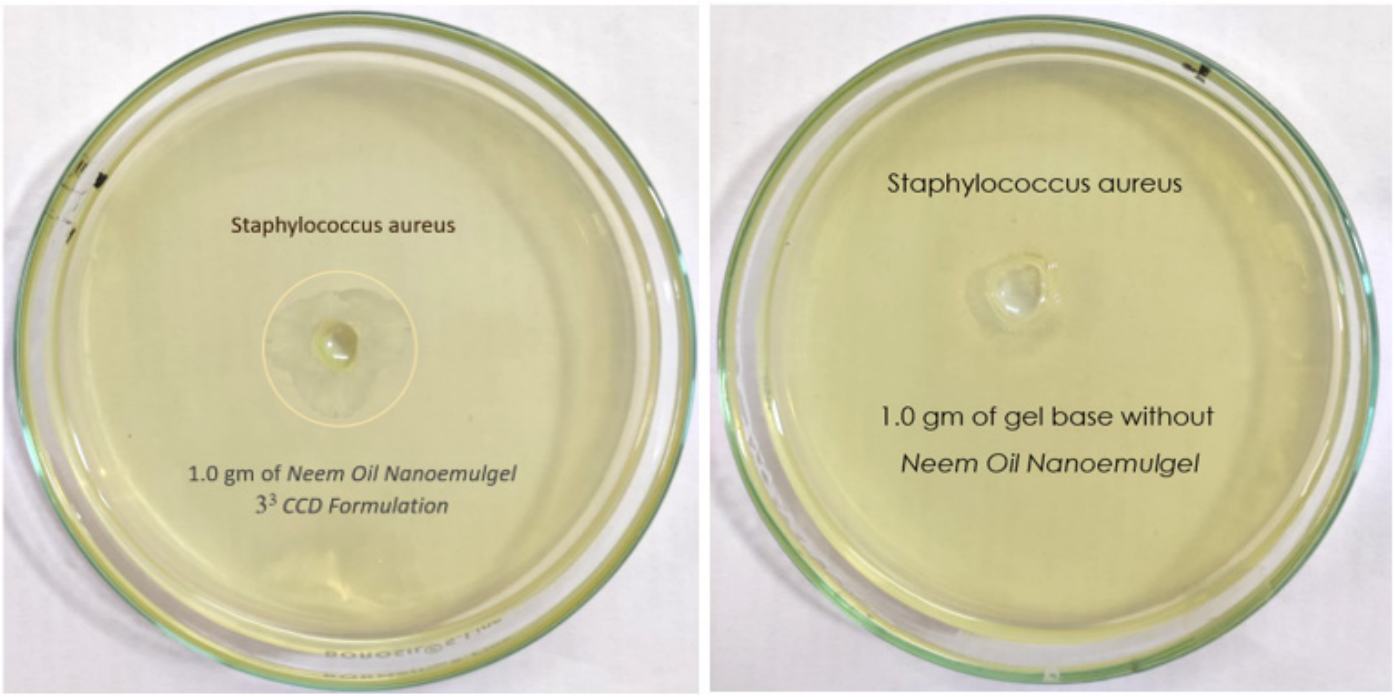

Figure 7: (A) Zone of Inhibition of *Neem* Oil Nanoemulgel formulation (B) Zone of Inhibition of gel base formulation without active ingredients.

0.512 to 0.612. This increase in droplet size may be attributed to crystallization during the freeze-thaw cycle, leading to the rupture of surfactant interfacial films, droplet coalescence, and phase separation between water and oil. The increase in PDI implies the accumulation of certain oil particles within the nanoemulsion.[30] However, the centrifugal stability remained unaffected, suggesting no phase separation even after centrifugation at 3000 rpm for 10 min. Despite the slight rise in oil particle size and PDI, the overall stability of the nanoemulsion formulation remained excellent throughout the freeze-thaw cycle. The findings from the *in vitro* antibacterial studies indicated notable antibacterial effectiveness of the *Neem* oil nanoemulgel against *S. aureus*. This underscores the efficacy of the optimized formulation in potentially treating inflammation through transdermal application.

CONCLUSION

Nanotechnology has emerged as a crucial tool for enhancing the delivery of essential oils, which offer diverse therapeutic benefits. However, challenges such as odor, viscosity, and stability can impede patient compliance with conventional formulations. Nanoemulgel formulations of *neem* oil prepared using a high-speed homogenization method and optimized via a 3-factorial CCD design, have shown promise *in vitro* antibacterial studies against *Staphylococcus aureus*, while also demonstrating favorable kinetic stability. This suggests that essential oils can be effectively incorporated into nanocarriers within topical gels, facilitating improved delivery and utilization. These findings are significant, particularly given the traditional use, safety, and cost-effectiveness associated with *neem* oil.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CCD: Central Composite Design; **RSM:** Response Surface Methodology; **ANOVA:** Analysis of Variance; **FTIR:** Fourier Transform Infrared Spectroscopy; **PDI:** PolyDIsepersity Index; **OFAT:** One-Factor-at-a-Time; **TEM:** Transmission Electron Microscopy; **RPM:** Revolutions per Minute; **SEM:** Scanning Electron Microscope; **JMP:** John's Macintosh Project; **ATR:** Attenuated total Reflectance.

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