Industrial Engineering Journal
ISSN: 0970-2555
Volume : 53, Issue 10, No.3, October : 2024

FORMULATION AND EVALUATION OF SILVER NANOPARTICLES LOADED NANOEMULSION USING HIBISCUS ROSA SINENSIS ESSENTIAL OILS

Neeraj Rani Shri Baba Mastnath Institute of Pharmaceutical Science and Research, Baba Mastnath University, Asthal Bohar Rohtak 124021, Haryana, INDIA
Bhagwati Devi Shri Baba Mastnath Institute of Pharmaceutical Science and Research, Baba Mastnath University, Asthal Bohar Rohtak 124021, Haryana, INDIA

Abstract:
In this study, the formulation and evaluation of nanoemulsion loaded with silver nanoparticles using *Hibiscus rosa-sinensis* essential oil was performed. A low-energy technique was used to develop the nanoemulsion. For the formulation surfactant, distilled water, and essential oil concentrations were optimized. The formulated nanoemulsion was characterized by analyzing its stability, globule size, and zeta potential. The results showed that the nanoemulsion was stable, with no phase separation or creaming observed. The nanoemulsion's globule size was within the desired range, indicating a uniform and stable formulation. Moreover, the zeta potential of the nanoemulsion indicated its stability over time. By performing the MTT assay the Cytotoxic Effects against cell line HaCaT was observed in the Optimized Sample (IC50 = 11.94 ± 0.08 µl/ml *i.e.* viable cells were found 50% at this used concentration). By the DPPH scavenging method, the IC50 value of the Optimized sample was found to be 5. 08 ± 0. µg/mL which indicates that the sample possesses good antioxidant potential. Additionally, the nanoemulsion containing silver nanoparticles' antibacterial efficacy was assessed against *Escherichia coli* and *Staphylococcus aureus*. The results demonstrated a noteworthy level of inhibitory action against *Escherichia coli* and *Staphylococcus aureus*, suggesting the potential of the nanoemulsion loaded with silver nanoparticles as an antibacterial agent. The sample of nanoemulsion also possessed a good antifungal activity against *C. albicans*.

1. Introduction

The tropical floral plant *Hibiscus rosa-sinensis*, also known as the Chinese hibiscus or Shoe flower, has been utilized extensively in traditional medicine due to its various therapeutic properties [1]. The plant's essential oils have demonstrated antibacterial, anti-inflammatory, and antioxidant properties, indicating their potential use in a range of medical and cosmetic applications. Essential oils are naturally occurring volatile molecules that are derived from different plant components, including leaves, flowers, stems, and seeds. They are distinguished by their unique aromatic qualities [2].
The medicinal benefits of Hibiscus rosa-sinensis's phytochemicals, including polyphenols, flavonoids, and anthocyanins, have been extensively studied [3]. The essential oils of *Hibiscus rosa-sinensis* are rich in these phytochemicals. These antioxidant and antimicrobial compounds may contribute to the therapeutic potential of *Hibiscus rosa-sinensis* essential oils [4][5].
Small droplet sizes, usually between 20 and 200 nanometers, define the class of emulsion-based drug delivery methods known as nanoemulsions [6]. These systems offer several advantages, including improved drug solubilization, enhanced permeability, absorption, and protection of the encapsulated active compounds from degradation [7]. The formulation and optimization of *Hibiscus rosa-sinensis* essential oil-loaded nanoemulsions [NE] can be a promising approach to harness the therapeutic potential of this natural resource. High- or low-energy techniques can be used to manufacture NEs. Small droplets are produced by low-energy means either by drastic temperature fluctuations or by a fine colloidal equilibrium between water, oil, and surfactant [8][9].
Intense disruption forces like high shear, ultrasonic, or high-pressure homogenization are used in high-energy techniques [10]. A low-energy technique for the development of nanoemulsions is the phase inversion temperature (PIT) method, which makes use of the temperature-dependent solubility of non-ionic surfactants [11][12]. Recent research has shown the successful formulation of nanoemulsions

using different essential oils, such as caffeic acid and essential oils from other plant sources [13]. An interfacial coating of surfactant and co-surfactant molecules stabilizes nanoemulsions, which are transparent or translucent dispersions of oil and water that are kinetically stable. They offer several advantages over conventional emulsions, including improved drug solubility, enhanced bioavailability, and increased stability [14][15].

Recently, there has been a boom in interest in formulating innovative formulations for a range of uses in industries like food, cosmetics, and medicine [16]. One such promising area of research is the use of nanoemulsions, which are colloidal systems consisting of finely dispersed droplets of one immiscible liquid (the oil phase) within another immiscible liquid (the aqueous phase) [17]. These nanoemulsions offer advantages such as improved stability, enhanced bioavailability, and controlled release of active ingredients [16]. Nanoemulsions have been widely studied for the delivery of various bioactive compounds, including essential oils, which are known for their antimicrobial, antioxidant, and anti-inflammatory properties. They have been used in various applications, such as food preservation, drug delivery, and topical formulations [17,18]. In this study, we aim to formulate and characterize a silver nanoparticle-loaded nanoemulsion using *Hibiscus rosa-sinensis* essential oil. This study seeks to explore the potential synergistic antibacterial properties of the essential oil extracted from *Hibiscus rosa-sinensis* flowers in combination with silver nanoparticles within a nanoemulsion system [19]. The formulation and characterization of silver nanoparticle-loaded nanoemulsion using *Hibiscus rosa-sinensis* essential oil is a novel approach in the field of nanoemulsion technology and holds great potential for various applications, such as antibacterial coatings, wound healing products, and antimicrobial food packaging [20][21]. The development of such a formulation has the potential to provide a more effective and sustainable solution for combating bacterial infections and preserving the quality and safety of various products in different industries. The formulation and characterization of silver nanoparticle-loaded nanoemulsion using *Hibiscus rosa-sinensis* essential oil is a novel approach that aims to harness the antimicrobial properties of both silver nanoparticles and *Hibiscus rosa-sinensis* essential oil for various applications. This study can potentially contribute to developing innovative and effective antibacterial formulations that can be utilized in different fields, benefiting human health and the environment [22]. The successful formulation and characterization of the silver nanoparticle-loaded nanoemulsion using *Hibiscus rosa-sinensis* essential oil can provide valuable insights into the potential synergistic effects of combining silver nanoparticles and *Hibiscus rosa-sinensis* essential oil within a nanoemulsion system. These insights can pave the way for developing advanced and sustainable antibacterial solutions with various applications, from healthcare to food preservation [23]. The utilization of *Hibiscus rosa-sinensis* essential oil in the formulation and characterization of a silver nanoparticle-loaded nanoemulsion holds promise for various industries looking for effective and eco-friendly antibacterial solutions. This approach not only harnesses the antimicrobial properties of both silver nanoparticles and *Hibiscus rosa-sinensis* essential oil but also offers a sustainable alternative to traditional antibacterial agents that may have negative environmental impacts. Moreover, Hibiscus rosa-sinensis essential oil adds another level of advantages to this formulation because this plant is known to have many pharmacological qualities, such as anti-inflammatory, antioxidant, anti-microbial, and anti-diabetic activities [24]. The combination of silver nanoparticles and *Hibiscus rosa-sinensis* essential oil in a nanoemulsion formulation has the potential to provide a multifunctional and versatile antibacterial solution with enhanced efficacy and reduced adverse effects [25]. This research can potentially contribute to the development of safer and more sustainable antibacterial products in various industries, addressing the growing concern over antibiotic resistance and environmental pollution caused by traditional antibacterial agents [26]. By integrating the beneficial properties of both silver nanoparticles and Hibiscus rosa-sinensis essential oil, this research aims to create a novel antibacterial formulation that can effectively combat bacterial infections while promoting sustainability and environmental well-being [27]. This research aims to explore the potential of silver nanoparticle-loaded nanoemulsion formulated with Hibiscus rosa-sinensis essential oil for its synergistic antibacterial effects, as well as its potential applications in

various industries, such as healthcare and food preservation [22]. The silver nanoparticle-loaded nanoemulsion incorporating *Hibiscus rosa-sinensis* essential oil offers a sustainable and effective antibacterial solution with wide-ranging applications, ranging from wound care to food packaging [28]. This innovative approach not only utilizes the therapeutic properties of Hibiscus rosa-sinensis essential oil but also leverages the antibacterial capabilities of silver nanoparticles, resulting in a synergistic effect for enhanced antibacterial activity [20]. The formulation of silver nanoparticles loaded nanoemulsion using *Hibiscus rosa-sinensis* essential oil presents a novel approach to harness the antibacterial properties of both silver nanoparticles and *Hibiscus rosa-sinensis* essential oil for various applications, including healthcare and food preservation [29]. Furthermore, the use of nanoemulsion as a delivery system for silver nanoparticles and essential oil provides advantages such as improved stability, increased solubility, and enhanced bioavailability [30]. The combination of silver nanoparticles and Hibiscus rosa-sinensis essential oil in a nanoemulsion formulation can potentially provide a multifunctional and versatile antibacterial product with enhanced efficacy and reduced adverse effects.

1. Materials and Methods

1.1. Material

Analytical grade compounds were used to formulate the nanoemulsion. All reagent solutions were prepared using distilled water. Distilled water (W) was prepared at the laboratory. Tween 80 and glycerol were bought from Pvt. Loba Chemie Ltd. Silver nanoparticles (AgNPs) were green synthesized in the laboratory (Rani N. and Devi B.). After being verified by a botanist, *Hibiscus rosa-sinensis* flowers that have been collected locally were used to extract essential oil.

1.2. Method

Extraction of *Hibiscus rosa sinensis* essential oil (H. oil): The hibiscus flowers contained some dirt and sticky substance, such as microscopic sand grains. To the greatest extent possible, the material was cleansed to remove dangerous substances. Fresh hibiscus flower samples were needed for this approach, and they had to be cleaned for 60 minutes in distilled water and drying the flowers by the oven at 70° C for 1h. After that, the particle size of the sample was reduced by crushing and grinding. The essential oil was extracted from the Clevenger apparatus using solvent n-hexane. The first sign of the oil droplets appears after about three hours of boiling. The extracted oil had a dark brown color. Before examination, the oil sample was dried over anhydrous sodium sulfate.

1.3. Formulation of Nanoemulsion

The silver nanoparticles loaded nanoemulsion was prepared using the phase inversion temperature method. This low-energy emulsification technique involves the spontaneous formation of a stable and uniform dispersion of the oil phase in an aqueous medium [14]. The formulation was optimized by varying the ratios of oil, surfactant, and water to achieve the desired physicochemical properties.

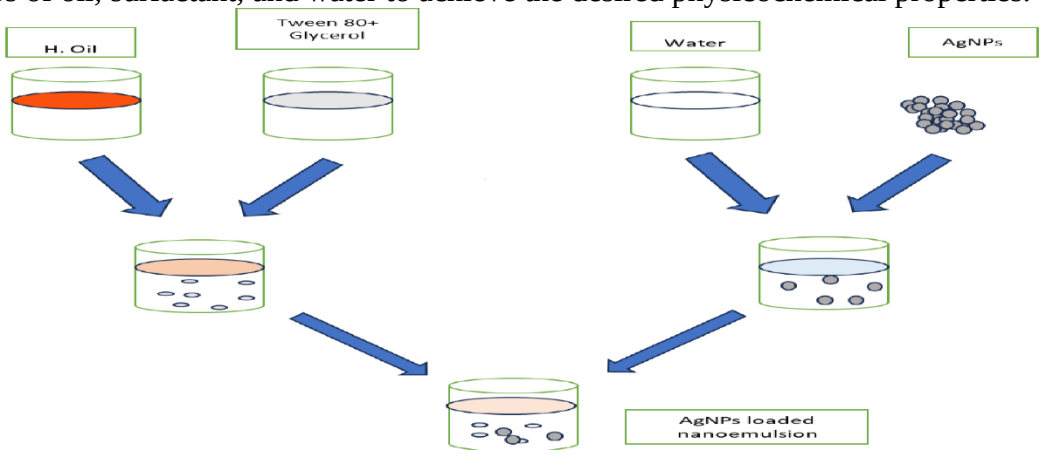

Fig. 1: Formulation of AgNPs loaded nanoemulsion using *Hibiscus rosa-sensis* oil

1.4. Optimization and Evaluation of Dependent Variables for Formulation of Nanoemulsion:

The formulation was optimized to achieve the desired droplet size, viscosity, and stability of the nanoemulsion. The ratio of oil to surfactant was a critical parameter that influenced the formation and stability of the nanoemulsion. Using the statistical program Design-Expert, ANOVA and their data analysis were employed to improve the synthesis parameters for nanoemulsion. Box Benken Design (BBD) suggested 12 experimental runs based on three variables at two levels. The amounts of tween 80 (% v/v), glycerol (% v/v), and oil (% v/v) were chosen as independent variables. The polydispersity index (PDI) and averaged droplet size (ADI) were the dependent variables. The developed model was evaluated using statistical techniques such as analysis of variance (ANOVA) or the F-test. R2, the coefficient of determination, served as an indicator of how well the model equation fit the data. The correlations between responses were displayed using actual versus predicted value, 3-D surface designs, and contour graphs.

1.5. Evaluation of Nanoemulsion

Determination of zeta potential, polydispersity index, and average droplet size (ADS) The Zeta sizer (Malvern Instruments), which operates based on dynamic light scattering (DLS) technique, was used to evaluate the average droplet size (ADS), polydispersity index (PDI), and zeta potential of all generated nanoemulsion formulations. The nanoemulsion formulations were tested on a Zeta sizer after being diluted ten times (1:10) with pure water. A disposable cuvette was used to collect the nanoemulsion for measurement. At a temperature of 25℃, the scattering angle was 90oC.

Stability of Nanoemulsion

To test for stability, the combined AgNps-loaded nanoemulsions were centrifuged for 20 minutes at 10,000 g. A month-long study was conducted by holding 50 milliliters of the nanoemulsions at three different temperatures: 25 ± 1 °C, 37 ± 1 °C, and 60 ± 1 °C [31]. The samples were checked for flocculation, creaming, and unstable phase separation.

Transmission Electron Microscopy (TEM) of Nanoemulsion

Transmission electron microscopy (TEM) was used to further examine the morphology and structure of the ideal nanoemulsion formulation. On a grid covered in carbon, the ideal nanoemulsion formulation was applied after being diluted ten times with distilled water. After that, 2% phosphotungstic acid was used to negatively stain the grids. Girds were then dried and subjected to an 80 kV accelerating voltage in a TEM examination. The measurements were conducted using the JEM-F200 from JEOL-New Delhi, which was run at an accelerating voltage of 200 kv.

In Vitro Cytotoxicity Evaluation of the Compounds-HaCaT Cell Line

MTT Assay

By using the MTT Assay, the cytotoxicity of the supplied sample was assessed on the adult human skin cell line HaCaT (obtained from NCCS Pune - Immortal keratinocyte cell line). 5000–8000 cells/well were grown in 96-well plates for 24 hours at 37°C with 5% CO2 in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L) supplemented with 10% FBS (Fetal Bovine Serum – HIMEDIA-RM 10432) and 1% antibiotic solution. Cells from various concentrations were treated the following day.

Following a 24-hour incubation period, the cell culture was treated with MTT Solution and incubated for an additional two hours. After the experiment was completed, the culture supernatant was discarded, and the cell layer matrix was dissolved in 100 µl of Dimethyl Sulfoxide (DMSO–SRL–Cat no.–67685). The results were measured at 540 nm and 660 nm using an Elisa plate reader (iMark, Biorad, USA). Utilizing Graph Pad Prism-6 software, IC-50 was computed. Pictures were taken with an AmScope digital camera (10 MP Aptima CMOS) under an inverted microscope (Olympus ek2).

DPPH Scavenging Assay

0.1 milliliter of 0.1 millipore DPPH solution was placed in a 96-well plate together with 5µl of an alternative stock of the test drug. The reaction process was set up in triplicate and the duplicate blanks were made with 0.2 ml of DMSO/Methanol and 5 µl of a chemical at various doses. The plate was left

in the dark for thirty minutes. Applying a microplate reader (iMark, BioRad), the decolorization was measured at 495 nm after the incubation.

The control was a reaction mixture with 20µl of deionized water in it. The activity of scavenging was displayed to the control as "% inhibition." Utilizing Software Graph Pad Prism 6, IC-50 was computed. DPPH Scavenging activity % = (Abs control - Abs sample/Abs control)×100.

Evaluation of anti-microbial activity

Using the Zone Inhibition Method (Kirby-Bauer method), the antibacterial activity was examined. *S. aureus*, *E. coli*, and bacterial culture (adjusted to 0.5 McFarland Unit - Approx cell density (1.5 X 108 CFU/mL)) were spread out in 100 µl on the MHA plates for inoculation. Next, discs containing 10 µl of various concentrations (0 to 100 mg/ml) were placed. A serial dilution process was used to obtain the necessary quantity for loading onto the disc by taking 10% of the samples.

Each plate had one disc filled solely with solvent, acting as a vehicle control, and one disc with 10µg of ciprofloxacin was used as a positive control. For twenty-four hours, the plates containing *S. aureus* and *E. coli* were incubated at 37 °C by Basil Scientific Corp. India. The disc's surrounding clear zones were measured and noted.

Anti-Fungal Activity

The Kirby-Bauer method, also known as the Zone Inhibition Method, was used to verify the antifungal activity. To inoculate the SDA plates, 100 µl of C. albicans fungal culture (adjusted to 0.5 McFarland Unit - Approx cell density (1.5 X 108 CFU/mL) was spread out. Next, discs containing 10 µl of various concentrations (0 to 100%/disc) were placed. Each plate had one disc filled solely with solvent, acting as the vehicle control, and one disc containing 50 µg of amphotericin B was employed as the positive control. The *C. albicans* plates were cultured for 24 hours at 37 °C in an incubator provided by Basil Scientific Corp. India. We measured and noted the clean zones that were formed surrounding the disk.

2. Results and Discussion

2.1. Optimization of AgNPs Loaded Nanoemulsion

To formulate the smallest globules with the lowest average droplet size (ADI), the statistical process optimization of AgNPs Loaded Nanoemulsion utilizing response surface methodology (RSM) was employed to determine the most prominent interaction between significant parameters. The effects of the independent factors on the dependent variables were examined using a BBD with three independent variables at different levels. The transformed values for each batch and their responses are shown in Tables 1 and 2. Figures 1 (a-d) and 2 (a-d) demonstrate the results. The statistical software's mathematical relationships were used to establish the relationship between the dependent and independent variables.

The following are the polynomial equations that were obtained:

$Y1 = 95.10 - 6.38\ A - 19.69\ B + 19.31\ C - 39.05\ AB + 4.70\ AC + 20.99\ BC - 17.52\ A^2 + 22.80\ B^2 + 37.27\ C^2$ (Equation 1)

$Y2 = 25.12 - 0.1514\ A - 1.82\ B + 0.3440\ C - 1.85\ AB + 0.0304\ AC - 0.6867\ BC$ (Equation 2)

Y1 represents the response, i.e., average droplet size (ADI), and Y2 represents the response poly dispersity Index (PDI). The variables are a, B, C, AB, AC, BC, A2, B2, and C2. Polynomial equations can be utilized to conclude taking into account the numerical sign and size of the coefficient. Average droplet size (ADI) and poly dispersity Index were found to have correlation coefficients (R2) of 0.93 and 0.88, respectively, indicating a satisfactory fit. The higher the correlation between experimental and expected responses, the closer the R2 number is to 1. A decent model's R2 should therefore fall between 0 and 1; the closer it is to 1, the better fit the model is considered to be [32]. The antagonistic influence of this variable on size is indicated by the negative values of coefficients A and B in Equation 1. When both coefficients A and B in equation 2 are positive, it means that these two variables have a positive effect on the polydispersity Index.

Table 1: Results of BBD for the Formulation of AgNPs Loaded Nanoemulsion

	Independent factors	Dependent factors

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Run	Amount of *Hibiscus rosa-sensis* oil (% v/v) (X1)	Tween 80 (% v/v) (X2)	Glycerol (% v/v) (X3)	Average Droplet Size (nm) (Y1)	Poly Dispersity Index (Y2)
1	10	10	5	98.58	25.2
2	15	15	7	117.53	20.6
3	10	15	7	175.78	23.3
4	15	5	5	153.55	28.5
5	10	15	2.5	95.19	23.8
6	10	5	2.5	176.55	26.3
7	10	10	5	96.84	24.1
8	5	10	7	135.84	25.8
9	5	5	2.5	131.05	24.2
10	15	10	7	132.49	26
11	5	15	5	130.53	25.1
12	10	15	2.5	95.19	23.8

Table 2: Summary of Analysis of Variance (ANOVA)

Source	**Df**	**Sum of Squares**	**Mean Square**	**F-value**	**p-value**	
Model	9	9486.93	1054.10	1392.66	0.0007	significant
A-Amount of *Hibiscus rosa-sensis* oil	1	40.64	40.64	53.69	0.0181	
B-Tween 80	1	382.61	382.61	505.50	0.0020	
C-Glycerol	1	164.71	164.71	217.61	0.0046	
AB	1	1495.16	1495.16	1975.37	0.0005	
AC	1	14.73	14.73	19.46	0.0477	
BC	1	256.98	256.98	339.51	0.0029	
A^2	1	165.68	165.68	218.89	0.0045	
B^2	1	178.45	178.45	235.76	0.0042	
C^2	1	1671.39	1671.39	2208.20	0.0005	
Pure Error	2	1.51	0.7569			
Cor Total	11	9488.45				
Std. Dev.	0.8700	**Mean**	128.26			
R^2	0.9998	**Predicted R^2**	NA(1)			
Adjusted R^2	0.9991	**Adeq Precision**	102.4430			

2.2. ANOVA Analysis Report

The significance and suitability of the model were evaluated using analysis of variance (ANOVA). Tables 2 and 3 contain statistical information such as standard error, F value, p-value, and the sum of squares. Average droplet size and polydispersity index are significantly influenced by the relevant variables, as shown in Table 2, 3 where a p-value of < 0.05 for the independent variables shows the model is significant. The predicted vs actual, 3D response surface and associated contour plots for average droplet size and polydispersity index are shown in Figures 2 (a-d), and Figure 3 (a-d).

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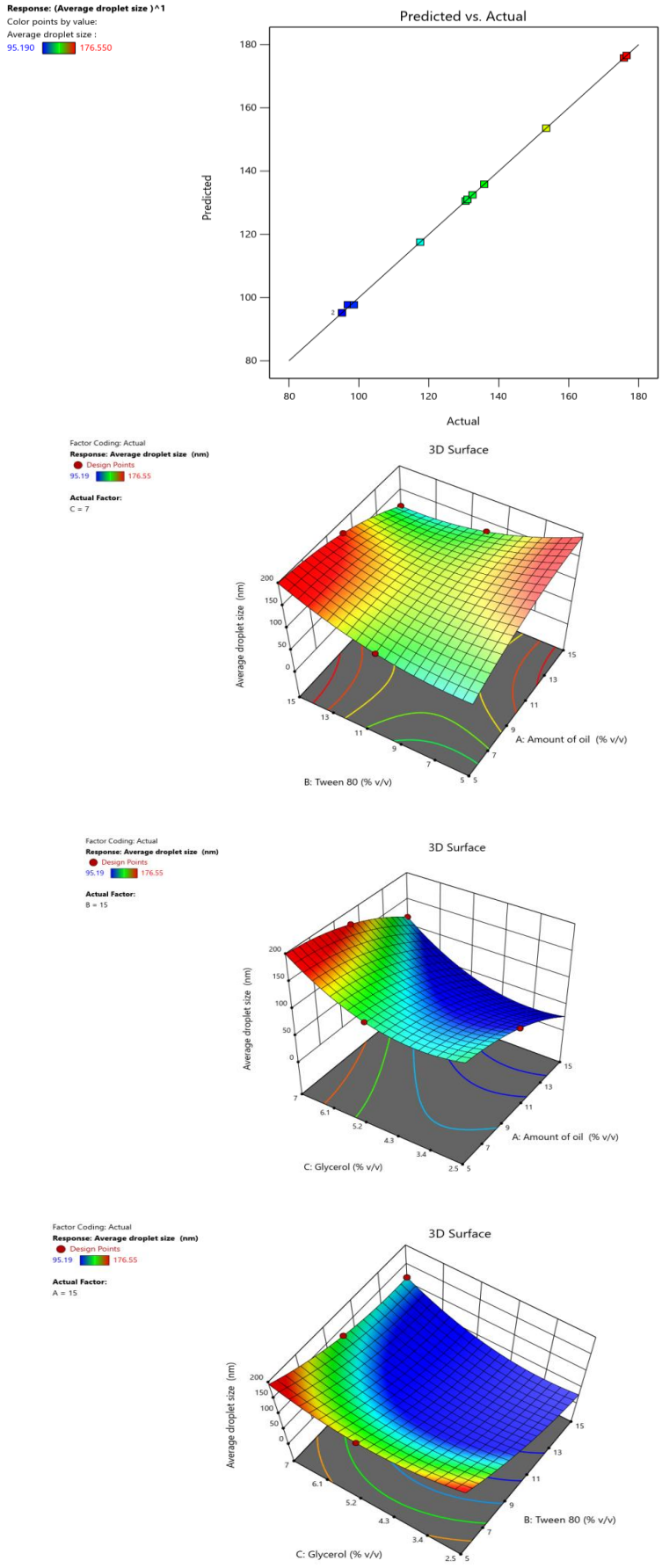

Figure 2: Graphs of Average Particle Size (a-d)

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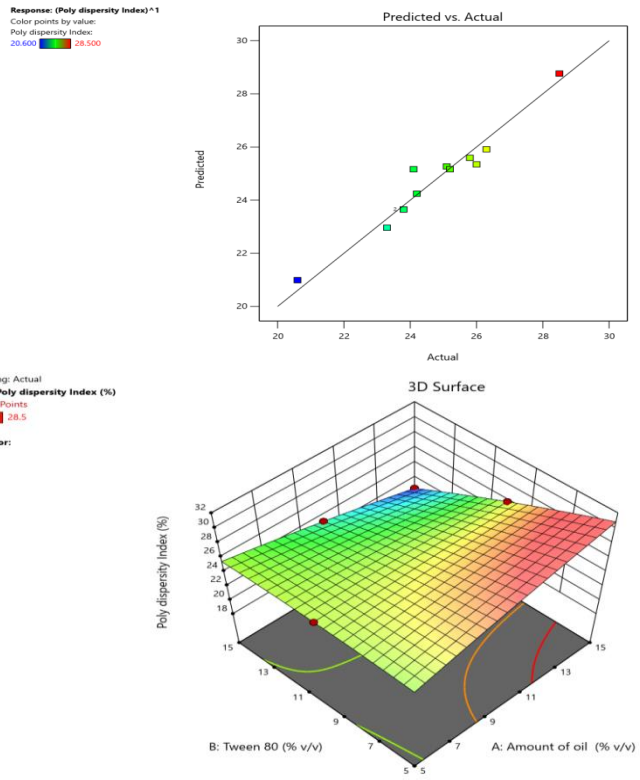
To generate the best nanoemulsion with its polydispersity index (PDI), the statistical process optimization of nanoemulsion formulation utilizing response surface methodology (RSM) was employed to determine the most prominent interaction between significant parameters. The effects of the independent factors on the dependent variables were examined using a BBD with three independent variables at different levels. The transformed values for each batch and their responses are shown in Table 1. Figure 2 (a-d) demonstrates the results. The statistical software's mathematical relationships were used to establish the relationship between the dependent and independent variables.

Table 3: Summary of Results of Analysis of Variance (ANOVA)

Source	df	Sum of Squares	Mean Square	F-value	p-value	
Model	6	39.14	6.52	15.09	0.0046	Significant
A-Amount of *Hibiscus rosa-sensis* oil	1	0.0729	0.0729	0.1687	0.6983	
B-Tween 80	1	17.82	17.82	41.24	0.0014	
C-Glycerol	1	0.5530	0.5530	1.28	0.3093	
AB	1	8.14	8.14	18.84	0.0074	
AC	1	0.0015	0.0015	0.0034	0.9560	
BC	1	1.29	1.29	2.99	0.1445	
Residual	5	2.16	0.4322			
Lack of Fit	3	1.56	0.5187	1.71	0.3890	not significant
Pure Error	2	0.6050	0.3025			
Cor Total	11	41.30				
Std. Dev.	0.6574	**Mean**	24.72			
R^2	0.9477	**Predicted R^2**	0.6777			
Adjusted R^2	0.8849	**Adeq Precision**	15.4819			



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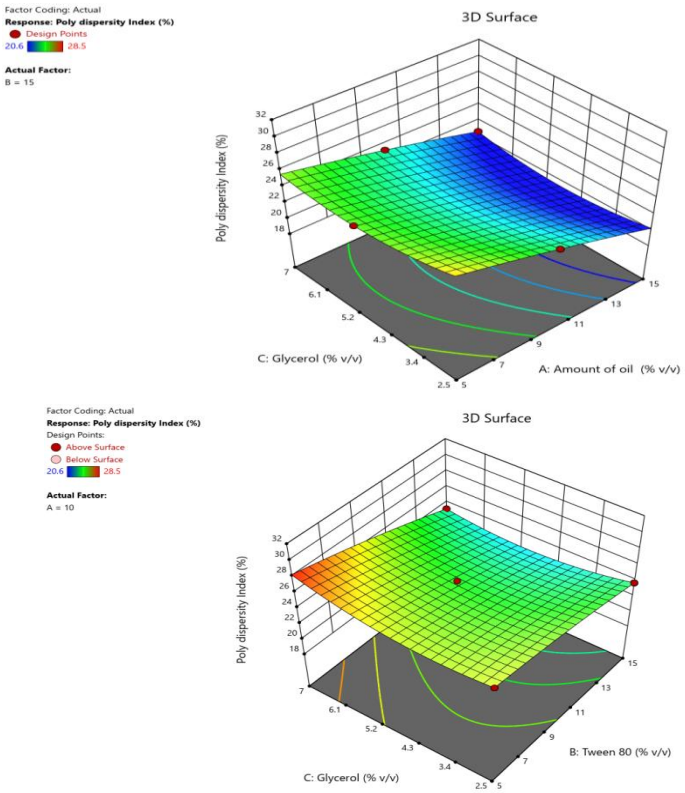

Figure 3: Graphs of Average particle size (a-d)

2.3. Optimized Formulation of AgNPs Loaded Nanoemulsion

Through numerical optimization, the batch with the least average Droplet Size (nm) and least ploy dispersity index value closer was chosen. The optimum conditions for formulated AgNPs loaded nanoemulsion came out to be the amount of oil (10%), Tween 80 (15%), and glycerol (2.5%). Additionally, it showed an anticipated response for the formulated AgNPs loaded nanoemulsion, which had an average Droplet Size of 95.19 and polydispersity index of 23.8. The obtained values were within 10% of the predicted response's error, demonstrating the mathematical models and selected design's good fit. The zeta potential of the optimized formulation was found to be -22.6 mV. The negative potential indicates a good stability of nanoemulsion.

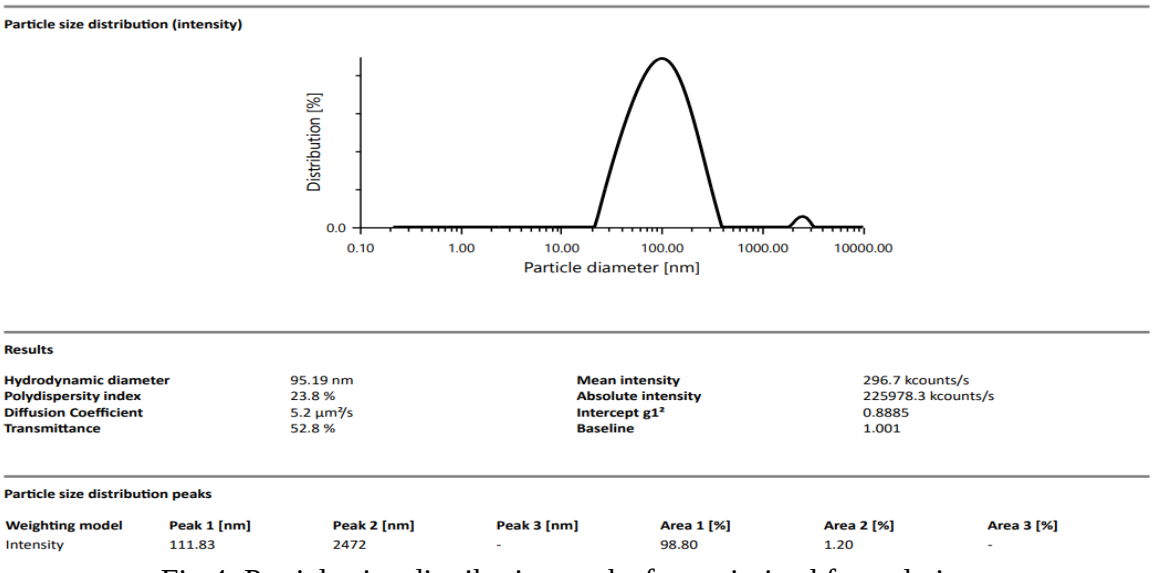

Fig.4: Particle size distribution peaks for optimized formulation

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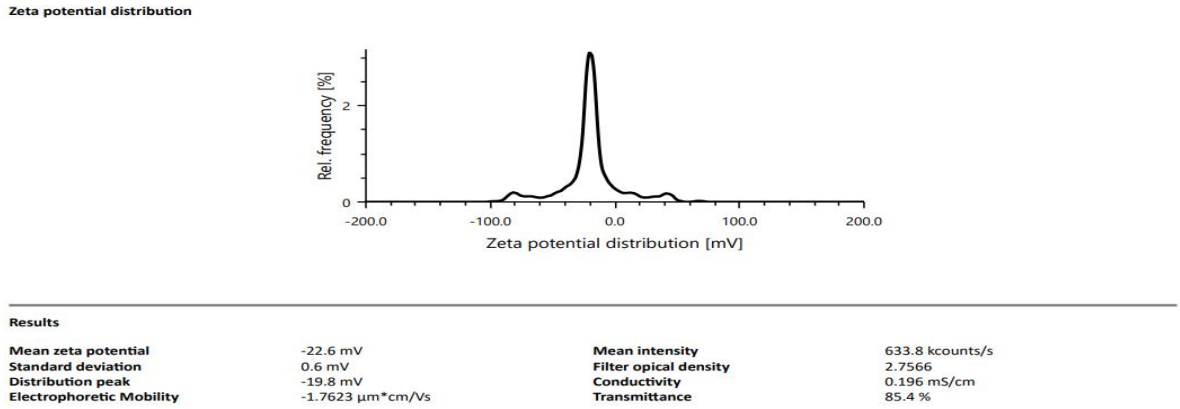

Fig.5: Zeta potential distribution of the optimized formulation

TEM analysis

A TEM analysis was conducted and recorded on an optimized sample of AgNP-loaded nanoemulsion. The optimized sample of AgNP-loaded nanoemulsion is nearly spherical, as seen by the TEM images. Figures 6(a) and 6(b) display the histogram of the particle size distribution with extract after any really small particles have been removed. This shows that nanoparticles range in size from 15 to 101 nm, with an average droplet size of 80.90 nm.

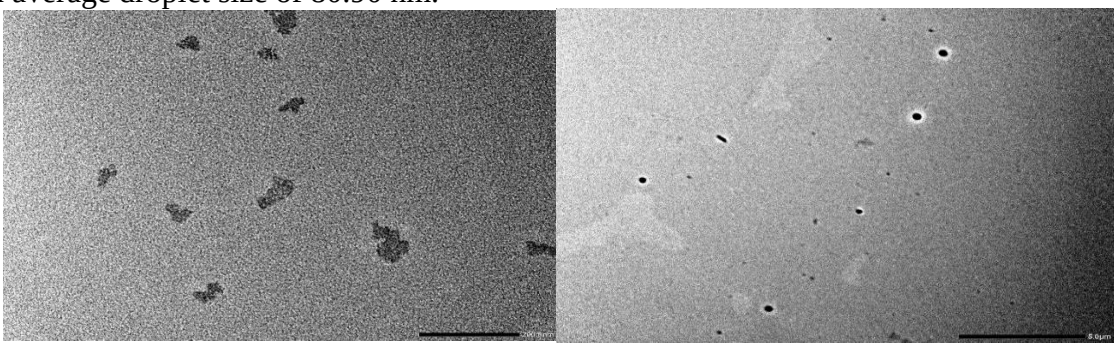

Fig.6 (a,b): TEM images of optimized sample nanoemulsion

MTT Assay

Cytotoxic Effects against cell line HaCaT were observed in the Optimized Sample (IC50 = 11.94 ± 0.08 µl/ml *i.e.* viable cells were found 50% at this used concentration). The positive control Ciprofloxacin displayed potent cytotoxicity against HaCaT cells at a 50 µg/mL concentration. The microscopic images showed that the cells in the treated group were round-shaped and detached from the culture surface compared to the control group.

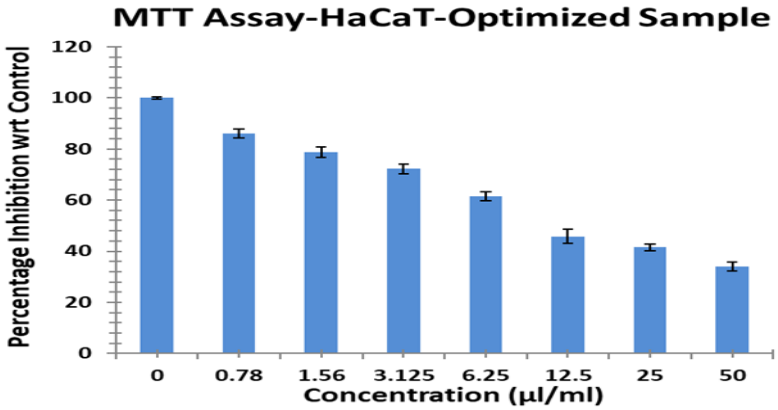

Fig. 7: Cytotoxic Effects against cell line HaCaT was observed in the Optimized Sample (IC50 = 11.94 ± 0.08 µl/ml i.e. viable cells were found 50% at this used concentration)

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DPPH Scavenging Assay

The antioxidant potential of the Optimized sample was evaluated by DPPH free radical scavenging assay. The results showed that the Optimized sample exhibited moderate DPPH radical scavenging activity at the tested concentrations. The IC50 value of the Optimized sample was found to be 5. 08 ± 0. µg/mL indicates that the sample possesses good antioxidant potential [34]**.** The results suggest that the Optimized sample could be a potential source of natural antioxidants.

In conclusion, the Optimized sample displayed promising *in vitro* cytotoxic effects against HaCaT cells.

2.4. *In Vitro* Cytotoxicity Evaluation of the Compounds-HaCaT

Anti-Microbial-Zone Inhibition Test

The antibacterial activity of the Optimized sample was evaluated against S. aureus and E coli in terms of the zone of inhibition. Based on the results obtained from the experimental work, when the test organism was treated with different amounts of samples on the agar plate then a clear zone of inhibition was formed. The zone of inhibition is an area around a disk on an agar plate where no bacterial growth is observed due to the presence of an antimicrobial agent. It is used to determine whether a particular test organism is susceptible to the action of a particular antimicrobial agent or not. With a clear zone of inhibition formed around the disks containing the Optimized sample, it can be concluded that the sample possessed good antibacterial activity against *S. aureus* and *E. coli.* [33].

Based on the study, it was observed that when test organism *S. aureus* was exposed to different concentrations of the sample the sample Optimized sample exhibited antimicrobial activity (MIC = Approx 62 µg/ml)**.** Concentration inhibiting at least 20% of bacterial growth is considered MIC.

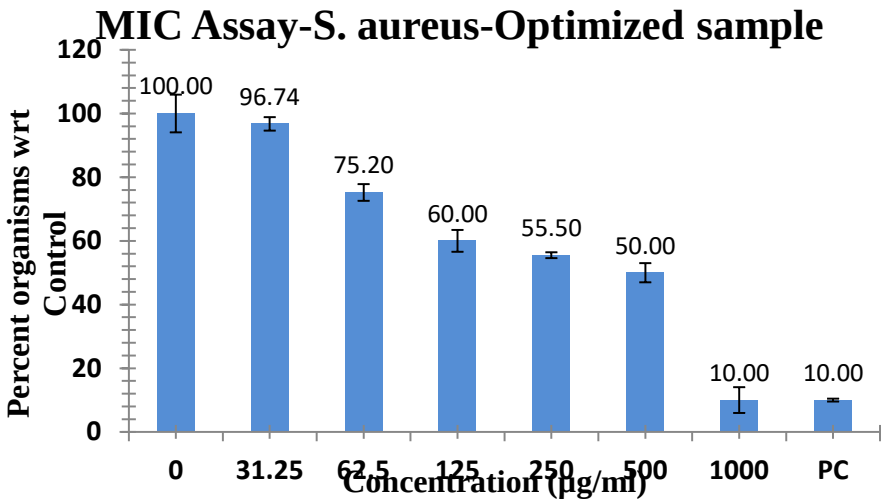

Fig.8: **MIC Assay-*S. aureus*-Optimized sample**

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ISSN: 0970-2555
Volume : 53, Issue 10, No.3, October : 2024

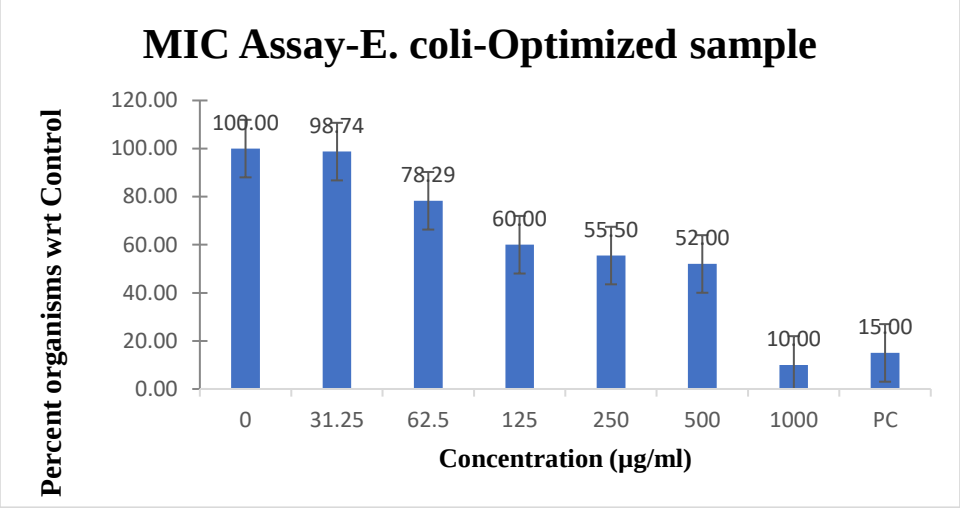

Fig.9: **MIC Assay-*E. coli*-Optimized sample**

Anti-Fungal Activity

The antifungal activity of the Optimized sample was evaluated against *C. albicans* in terms of the zone of inhibition. Based on the results obtained from the experimental work, when the test organism was treated with different amounts of samples on an agar plate, a clear zone of inhibition was formed around any disk except positive control. The zone of inhibition is an area around a disk on an agar plate where no fungal growth is observed due to the presence of an antimicrobial agent. It determines whether a particular test organism is susceptible to a particular antimicrobial agent's action. With a clear zone of inhibition formed around the disks containing the Optimized sample, it can be concluded that the sample has possessed an antifungal activity against *C. albicans*. Based on the study, it was observed that when test organism *C. albicans* was exposed to different concentrations of the sample the sample Optimized sample exhibited good antifungal activity (MIC = Approx 1000 µg/ml). Concentration inhibiting at least 20% of fungal growth is considered MIC.

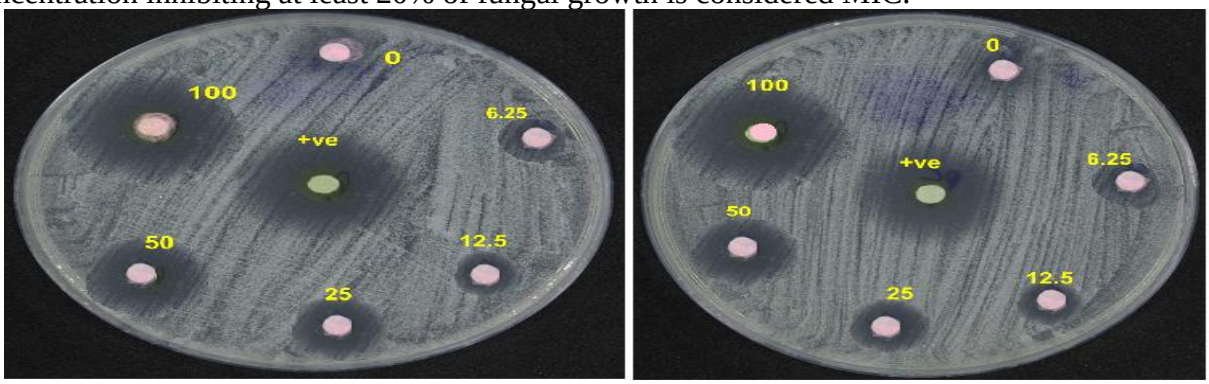

Fig.10: Images showing the zone of inhibition at different concentrations of optimized nanoemulsion sample with various concentrations (0-100 mg/mL) with positive control

3. CONCLUSION

The present study formulated a silver nanoparticle-loaded nanoemulsion using *Hibiscus rosa-sinensis* essential oil with a low-energy technique. The formulation was optimized using the BBD method and the optimized sample was evaluated for various parameters. The nanoemulsion exhibited an average size of 95.19 nm by zeta sizer. The negative zeta potential indicates the stability of the nanoemulsion with no phase separation or creaming. By performing the MTT assay the Cytotoxic Effects against cell line HaCaT were observed in the Optimized Sample (IC50 = 11.94 ± 0.08 µl/ml *i.e.* viable cells were found 50% at this used concentration). By the DPPH scavenging method, the IC50 value of the Optimized sample was found to be 5. 08 ± 0. µg/mL which indicates that the sample possesses good antioxidant potential. Additionally, the nanoemulsion containing silver nanoparticles' antibacterial

efficacy was assessed against *Escherichia coli* and *Staphylococcus aureus*. The results demonstrated a noteworthy level of inhibitory action against *Escherichia coli* and *Staphylococcus aureus*, suggesting the potential of the nanoemulsion loaded with silver nanoparticles as an antibacterial agent. The sample of nanoemulsion also possessed a good antifungal activity against *C. albicans*.

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