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Formulation development and optimization of nanoemulsion gel containing *Prosopis cineraria*, *Aerva javanica*, and *Fagonia indica* extracts for treatment of arthritis

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ABSTRACT

Herbal extracts are interesting therapeutic candidates because they are enriched with numerous bioactive compounds which can be used to treat inflammatory disorders, such as rheumatic pain, inflammation, and arthritis. However, numerous issues are associated with the use of bioactive compounds, such as poor solubility, less permeability, confined bioavailability, and instability due to oxygen and light. In the present study, formulation and optimization of nanoemulsion containing *Prosopis cineraria*, *Aerva javanica,* and *Fagonia indica* hydroalcoholic extracts (0.2% w/w) was performed by the ultrasonication method. The response surface methodology was employed to optimize the nanoemulsion by using Box–Behnken experimental design. The oil concentration (oleic acid and olive oil), surfactant, and cosurfactant concentration (Tween 80 and soya lecithin) were three independent variables, and droplet size and % transmittance were two dependent variables. The droplet size, transmittance, polydispersity index, and zeta potential of optimized nanoemulsion formulation were 70.72 nm, 99.21%, 0.259, and −15.9 mV, respectively. The Fourier transform infrared analysis revealed that there was no interaction between the plant extracts and excipients. Hence, it can be concluded that these results will help in the design of nanoemulsion with optimum independent variables.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, destructive disease of the joints induced by synovial membrane inflammation due to infiltration of inflammatory cells into the synovial tissue of joints, accompanied by swelling, pain, and deformity in the affected joints [1]. Over 60 million adults worldwide are affected by this disease, and its prevalence is three times more in women than in men. In India, studies reported a prevalence of RA from 0.28% to 0.7% for total study populations [2]. The use of non-steroidal anti-inflammatory drugs in the treatment of RA is associated with severe adverse effects, including gastrointestinal bleeding, nephrotoxicity, and cardiovascular adverse effects [3]. Since ancient times, bioactive extracts have been utilized in herbal formulations due to their excellent therapeutic properties to treat various health ailments, including RA, because of the side effects of synthetic drugs, as they have the potential to combat the inflammatory mediators of this autoimmune disease. *Prosopis cineraria*, *Fagonia indica*, and *Aerva javanica* are traditionally used as ethnomedicine in India, Pakistan, and Africa for the treatment of inflammatory disorders such as rheumatic pain, inflammation, and arthritis [4–7].

Literature survey reveals that *A. javanica, P. cineraria,* and *F. indica* exhibited significant analgesic, anti-inflammatory, and immunomodulatory activities [5,8–10]. However, numerous issues are associated with the use of bioactive extracts, such as insufficient water solubility, low skin penetration, and limited bioavailability [11,12]. These obstacles regarding the use of natural extracts raise the necessity of novel nanoformulation, which offers stability and exploits the therapeutic potential of plant extracts. The application of nanoemulsion loaded with

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phytoconstituents has excellence such as small droplet size (20–500 nm), significant solubility and excellent permeability, protection of drug candidates, and enhanced bioavailability, thereby reducing the dose and, hence, side effects. However, due to the low viscosity of nanoemulsion, it has low retention on the skin. Therefore, nanoemulsions are often incorporated into a gel system. Based on the literature review, oil concentrations of 10%–15% w/w could be easily emulsified, resulting in small droplet sizes with no phase separation. Previous studies have indicated that high levels of surfactants, especially ionic surfactants, can cause irritation [13]. Therefore, in the preparation of nanoemulsions, nonionic and natural surfactants were selected at the lowest possible concentrations. In this study, the selection of formulations was based on the criterion of using the minimum concentration of Smix in the formulation.

The topical route of administration has received much attention in the drug delivery domain because of the more flexibility in the design of dosage forms in comparison to other drug delivery designs for other routes. In conclusion, this evolves the concept to local dermal sustained drug delivery and led to the development of nanoemulsion [14]. These selected plants of arid region are used topically as a remedy to treat inflammatory disorders. This is evident from the results of previous studies that *P. cineraria*, *F. indica*, and *A. javanica* possess anti-inflammatory activity. Literature reveals that numerous plant extract-loaded nanoemulsion have been developed. However, no attempt has been made to formulate and characterize the *P. cineraria*, *F. indica*, and *A. javanica* extracts containing nanoemulsions. Therefore, by keeping this fact in view, the present research work was conceptualized and performed with the following objectives: (i) formulation and optimization of *P. cineraria*, *F. indica*, and *A. javanica* extract-loaded nanoemulsion; (ii) characterization and stability studies of optimized nanoemulsion; and (iii) evaluation of *in-vitro* anti-inflammatory activity of nanoemulsion.

2. MATERIALS AND METHODS

2.1. Chemicals

Oleic acid, olive oil, and Tween 80 were purchased from Modern Scientific Store (Gwalior, India). Soya lecithin (HiMedia Laboratories) was received as gift sample. Deionized water was used during all experiments. All of these chemicals were of analytical grade.

2.2. Plant authentication

The plants *P. cineraria*, *A. javanica*, and *F. indica* were collected from the natural habitat, Shikargarh area of the Jodhpur district in Rajasthan, India, in February 2024. The plant material was authenticated by a senior scientist and the Head of Office Sriman Lal Meena of Botanical Survey of India, Jodhpur, Rajasthan. A voucher specimen was prepared and deposited in the institute herbarium under reference no. BSI/AZC/I/2012/Tech/2023-24 (Pl. Id.)/645.

2.3. Preparation of hydroalcoholic extract

Prosopis cineraria bark, *A. javanica* leaves, and *F. indica* whole plant were collected, washed thoroughly with running tap water, dried in the shade, and coarsely powdered using a mixer grinder. Then, a 3 l conical flask, separate for each plant, was filled with about 100 g of air-dried plant material. Next, 1,000 ml of hydroalcoholic solution—ethanol: water (70:30) was added, and the powdered material was macerated for 72 hours at room temperature. After 72 hours, all three plant extracts *P. cineraria* hydroalcoholic extract (PCHE) *F. indica* hydroalcoholic extract (FIHE), and *A. javanica* hydroalcoholic extract (AJHE) were filtered through Whatman filter paper No. 1. The filtrate was collected and evaporated using a water bath. The dried extracts were kept refrigerated at 4°C in airtight containers until needed [15–17].

2.4. Fourier transform infrared spectroscopy of plant extract

Fourier transform infrared (FTIR) spectrophotometry was done by using the PerkinElmer (FTIR Spectrometer Spectrum TWO DTGS, S. No-105627). The FTIR spectrophotometer was used to evaluate the functional groups of bioactive components in plant extract. All samples were loaded in an FTIR spectroscope, with a scan range from 400 to 4,000 cm^{-1} with a resolution of 4 cm^{-1} [18,19].

2.5. Preparation of plant extract-loaded nanoemulsion

Nanoemulsion was prepared using the ultrasonication method described by Ahmad *et al.* [20], Mehmood and Ahmed [21], and Nirmala *et al.* [22], with slight modification. The nanoemulsion was composed of 10%–15% w/w oil phase, 1%–3% w/w soya lecithin, and 1%–3% w/w Tween 80. Briefly, for oil phase preparation, 70% oleic acid and 30% olive oil were mixed together. All extracts, each of 200 mg, that is, PCHE, AJHE, and FIHE (0.2% w/w), were dissolved in the oil phase and mixed thoroughly by using a magnetic stirrer. Tween 80 (nonionic surfactant) and soya lecithin (natural cosurfactant) act by reducing interfacial tension between oil and aqueous phase. Tween 80, a nonionic surfactant generally recognized as safe for nanoemulsion formulation, because ionic surfactants have been reported for skin irritation and discomfort [18]. Therefore, in the present study, the nonionic surfactant Tween 80 and natural cosurfactant soya lecithin were selected and dissolved in the oil phase. The aqueous phase was added dropwise with continuous stirring at 600 rpm for approximately 30 minutes to form a coarse emulsion. The resulting coarse emulsion was then subjected to ultrasonication for the formation of nanoemulsion using a probe sonicator (LABMAN Probe Sonicator PRO650) at 60% power with a 5.0-s pulse rate for 20 minutes. During the sonication process, the sample-containing beaker was kept in an ice bucket to regulate the temperature increase. The nanoemulsion was equilibrated at 25°C–30°C before characterization.

2.6. Optimization of plant extract nanoemulsion by using Box–Behnken design

Response surface methodology was conducted by using Design Expert software (version 13.0.5, Stat-Ease, Minneapolis, MN). A Box–Behnken design was used to study the effect of the independent variable on the dependent variable. The independent variables were concentration of oil (% w/w), concentration of Tween 80 (% w/w), and concentration of soya lecithin (% w/w). Dependent variables were droplet size (nm) and transmittance (%). The study range and level are shown in Table 1. A total of 15 runs were obtained with three center

Table 1. Independent variable with their coded and real values alongside dependent variables and their constraints in the Box–Behnken design.

Independent variable	Levels		
	Low	Intermediate	High
	−1	0	+1
A = Concentration of oil (% w/w)	10	12.5	15
B = Concentration of Tween 80 (% w/w)	1	1.75	2.5
C = Concentration of soya lecithin (% w/w)	1	2	3
Dependent variable	**Constraints**		
Y_1 = Droplet size (nm)	Minimum		
Y_2 = Transmittance (%)	Maximum		

points. An optimized formulation was selected on the condition that it led to minimal droplet size and maximum transmittance.

2.7. Droplet size analysis

The droplet size of nanoemulsion was determined by the dynamic light scattering method by using Litesizer 500, which analyzes light scattering fluctuations due to particle Brownian motion. The nanoemulsion sample was properly diluted with double-distilled water to avoid particle interactions. The diluted sample was filled in the cuvette (Omega cuvette Mat. No. 225288) and examined for droplet size. All the measurements were conducted at 25°C, and each examination was made with three readings per sample [23,24].

2.8. Transmittance as response parameter

For determining the % transmittance, the Shimadzu UV spectrophotometer (UV-1800 Pharma Spec, Shimadzu, Japan) was used. One milliliter sample of the formulation was diluted 100-fold using distilled water. The diluted sample was placed in a UV spectrophotometer, and absorbance was checked at a 650 nm wavelength [25].

2.9. Statistical analysis

The response surface methodology of Box–Behnken design has been carried out with the response variables Y_1 droplet size and Y_2 transmittance. The analysis of variance (ANOVA) involved evaluating statistical parameters such as f ratio, *p* ratio, degree of freedom, and lack of fit for the interaction between independent variables [26]. The main goal was to obtain the most effective formulation with the minimum droplet size and maximum transmittance. The optimal model was selected from the experimental data. If the *p* values were below 0.05, then the model was considered statistically significant [27]. The model's fitness was determined by evaluating the coefficient of variation, determining the coefficient values, and adjusting the R^2 and projected R^2 values. The nanoemulsion was formulated using the most stable solution, resulting in a minimum droplet size (nm) and maximum transmittance (%).

2.10. Characterization

2.10.1. Drug-excipient interaction

FTIR analysis was conducted to investigate the interactions among the various components of the plant extract-loaded nanoemulsions, utilizing a PerkinElmer (FTIR Spectrometer Spectrum TWO DTGS, S. No-105627). FTIR profiling on PCHE, FIHE, AJHE, oleic acid, olive oil, Tween 80, and soya lecithin has been done. All samples were placed in the chamber and then scanned with a range of 400 to 4,000 cm^{-1} and then analyzed [18].

2.10.2. Microscopic evaluation of the nanoemulsion

The microscopic evaluation was carried out to identify the nature of the dispersed phase and continuous phase. In this study, plant extract-loaded nanoemulsion was mixed with methylene blue, which was got solubilized in the polar aqueous phase. The sample was dispersed on the glass slide and covered with a coverslip. Now, the sample was evaluated under the light microscope (Olympus CH20i) for the type of nanoemulsion formed. When observed under a microscope, the droplet should be colorless in a blue background, which indicates that the oil phase is the dispersed phase and the aqueous polar phase is the continuous phase. Thus, it confirms that the nanoemulsion was an oil-in-water type [28].

2.10.3. Evaluation of zeta potential and polydispersity index

Zeta potential and polydispersity index were evaluated by using Litesizer 500. The nanoemulsion sample was properly diluted with double-distilled water to avoid particle interactions. The diluted sample was filled in the cuvette (Omega cuvette Mat. No. 225288) and examined for droplet size. All the measurements were conducted at 25°C, and each examination was made with three readings per sample [29–31].

2.10.4. Thermodynamic stability study

A centrifugation test was performed to examine the thermodynamic stability of the nanoemulsion. Briefly, the nanoemulsion sample was filled in the centrifugation tube and centrifuged for 30 minutes at 3,500 rpm. After that, the nanoemulsions were visually observed for any phase separation [32]. Similarly, heating cooling cycle was performed to evaluate the thermodynamic stability. The nanoemulsion sample was stored at 4°C and 45°C temperatures for at least 48 hours, and the stability of the nanoemulsion was observed [27]. In the dilution test, 1 ml of the nanoemulsion sample was diluted 10 times with water to examine any phase separation in the nanoemulsion [25].

2.11. Preparation of plant extracts loaded nanoemulsion gel

For the preparation of nanoemulsion gel, 1 g of Carbopol 934 was added to 100 ml of distilled water and stirred for 30 minutes using a magnetic stirrer. Now add 0.5 ml of triethanolamine and 0.1 ml of dimethyl sulfoxide. After that, the plant extract-loaded nanoemulsion was incorporated in the gel with continuous stirring. Nanoemulsion gel was stored in a tight container [33].

2.12. Evaluation of nanoemulsion gel

The prepared nanoemulsion gel was visually inspected for color, homogeneity, presence of particles or aggregates, and physical appearance. pH of the gel was measured at room temperature. For pH measurement, 2 g of gel was dispersed in 20 ml of water, and then, pH was examined [34]. To evaluate

spreadability, 0.5 g of the freshly prepared nanoemulsion gel was positioned between two glass slides. A fixed weight of 500 g was applied on top of the slides for 1 minute. After removing the weight, the diameter of the area covered by the gel was measured, and this value was used to determine the spread ability.

2.13. Stability studies

Nanoemulsion undergoes instability over a period of time. Instability can lead to increased droplet size, phase separation, and creaming in nanoemulsions. Nanoemulsion was prepared and stored at 4°C ± 1°C and 25°C ± 1 °C for 1 month. After 1 month of storage, the nanoemulsion was examined for its physical characteristics, including droplet size, phase inversion, and creaming [35,36].

2.14. *In vitro* anti-inflammatory activity

2.14.1. Protein denaturation assay

Inhibition of protein denaturation assay was used to analyze the anti-inflammatory activity of nanoemulsion and compare it with the standard, earlier described by Banerjee *et al.* [37] and Ameena *et al.* [38], with slight modification. Briefly, 1 ml of PCHE, AJHE, FIHE, a nanoemulsion of plant extract, and diclofenac sodium at various concentrations of 100, 200, 500, and 1,000 µg/ml were mixed with 1 ml of a 5% aqueous solution of bovine serum albumin. A few drops of 1N hydrochloric acid were used to maintain a pH of 6.3 in the solution. Then, the samples were incubated at 27°C for 20 minutes, and after incubation, the samples were heated to 55°C for 30 minutes. After cooling, the samples were evaluated for turbidity at 660 nm using a spectrophotometer [37,38]. The experiments were performed in triplicate.

Percentage inhibition of protein denaturation was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control-Absorbance of Sapmle}}{\text{Absorbance of Control}} \times 100$$

3. RESULTS

3.1. FTIR analysis

The FTIR spectrum was used to identify the functional groups of the active components present in the extract. When

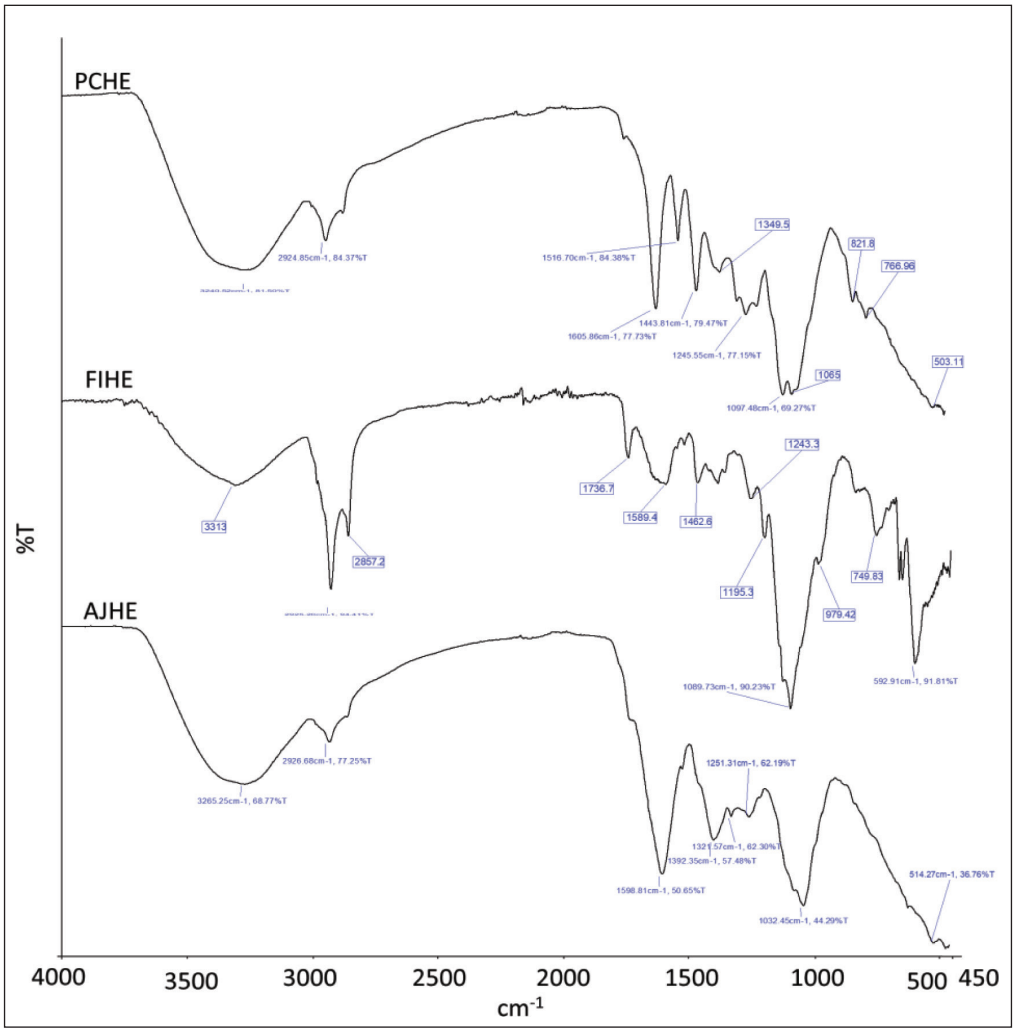


Figure 1. FTIR spectra of plant extracts.

the extract was passed into the FTIR, based on their peak ratio, functional groups were separated [39]. FTIR spectra of PCHE, FIHE, and AJHE obtained in the range 4,000−400 cm^{-1} are shown in Figure 1. The results of FTIR analysis confirmed the presence of O-H, CH, CH_2, CH_3, C=C-C, C-O, Si-O-Si, RONO, and $RONO_2$.

3.2. Box–Behnken design analysis

A total of 15 runs were conducted in order to check the effect of the concentration of oil, concentration of Tween 80, and concentration of soya lecithin on the dependent variable. The recorded response is found in Table 2. The effect of independent variables and their combinations on the estimated response, statistical parameters, and model fitness was examined using Design Expert 13 software. The ANOVA test validated the selected model at a confidence level of 95% and a *p*-value below 0.05 [40].

3.2.1. Effect of independent variable on average droplet size

After examining the effect of independent variable on the droplet size as indicated in Table 2, the droplet size varies between 72.94 and 163.95 nm, with an average droplet size of 123.92 nm. Statistical analysis was done by analysis of variance. ANOVA selects the quadratic model for the determination of significant value, lack of fit, and multiple correlation coefficient.

Table 2. Experimental design in coded and real variables, their droplet size, and transmittance.

Run	Coded variable			Real variable			Response variable	
	A	B	C	A: Concentration of oil (%v/v)	B: Concentration of Tween 80 (%v/v)	C: Concentration of soya lecithin (%w/w)	Droplet size (nm)	Transmittance (%)
1	−1	−1	0	10	1	2	130.94	98.99
2	1	−1	0	15	1	2	162.48	88.08
3	−1	1	0	10	2.5	2	77.69	99.59
4	1	1	0	15	2.5	2	131.46	95.74
5	−1	0	−1	10	1.75	1	107.65	54.78
6	1	0	−1	15	1.75	1	132.34	94.35
7	−1	0	1	10	1.75	3	72.94	93.64
8	1	0	1	15	1.75	3	145.93	43.62
9	0	−1	−1	12.5	1	1	163.95	99.48
10	0	1	−1	12.5	2.5	1	124.82	14.54
11	0	−1	1	12.5	1	3	144.43	2.37
12	0	1	1	12.5	2.5	3	103.46	98.99
13	0	0	0	12.5	1.75	2	113.69	95.18
14	0	0	0	12.5	1.75	2	126.29	98.99
15	0	0	0	12.5	1.75	2	120.8	97.78

Table 3. ANOVA of Box–Behnken design for droplet size.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value	
Model	9,561.79	9	1,062.42	35.43	0.0005	Significant
A-Concentration of oil	4,185.67	1	4,185.67	139.59	<0.0001	
B-Concentration of Tween 80	3,377.19	1	3,377.19	112.63	0.0001	
C-Concentration of soya lecithin	480.50	1	480.50	16.02	0.0103	
AB	123.54	1	123.54	4.12	0.0981	
AC	583.22	1	583.22	19.45	0.0070	
BC	0.8464	1	0.8464	0.0282	0.8732	
A^2	182.67	1	182.67	6.09	0.0567	
B^2	569.22	1	569.22	18.98	0.0073	
C^2	8.18	1	8.18	0.2729	0.6237	
Residual	149.93	5	29.99			
Lack of fit	70.11	3	23.37	0.5856	0.6802	Not significant
Pure error	79.82	2	39.91			
Cor total	9,711.71	14				

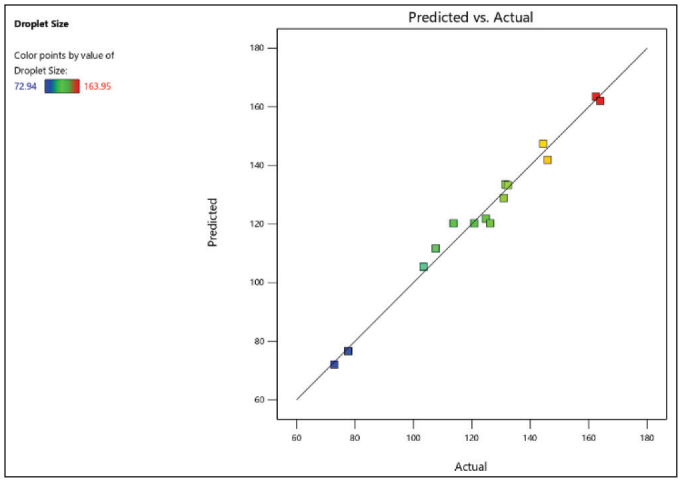


Figure 2. Graph between predicted and actual droplet size.

The response variable in the model had a *p*-value of 0.0005, which is much less than the required level of significance. The model's significance is validated by the *p*-value [41]. The lack of fit value in our model was 0.5856, indicating a non-significant *p*-value (Table 3). Based on the model's lack of fit and *p*-value [27], we can observe that the quadratic model adequately describes the response. The *F* value also confirms the model's fitness. Variables A and B show the higher *F* value, which shows a higher effect on the response variable. Variable C and the combination of variables also show significant effects on droplet size. The combination of variable BC has a very low *f* value, which indicates non-significant effects on droplet size.

The effect of the independent variable on the response variable is also displayed by the R^2 value that was generated from the ANOVA. The model's fitness is also indicated by the greater R^2 score. The obtained R^2 value in this study is 0.9846% or 98.46%. The chosen model had a predicted R^2 value of

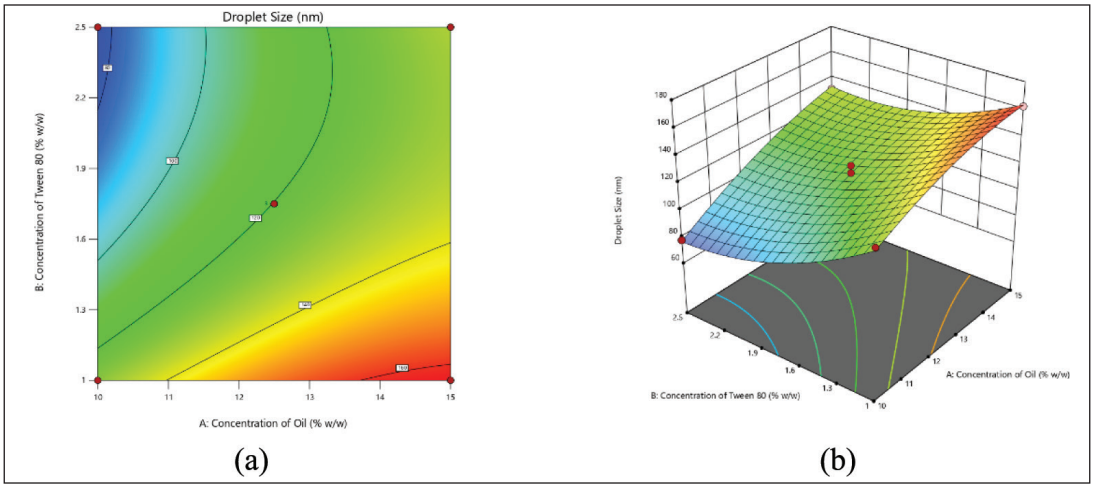


Figure 3. Effect of concentration of oil and concentration of Tween 80 on droplet size: (a) contour plot and (b) 3D surface plot.

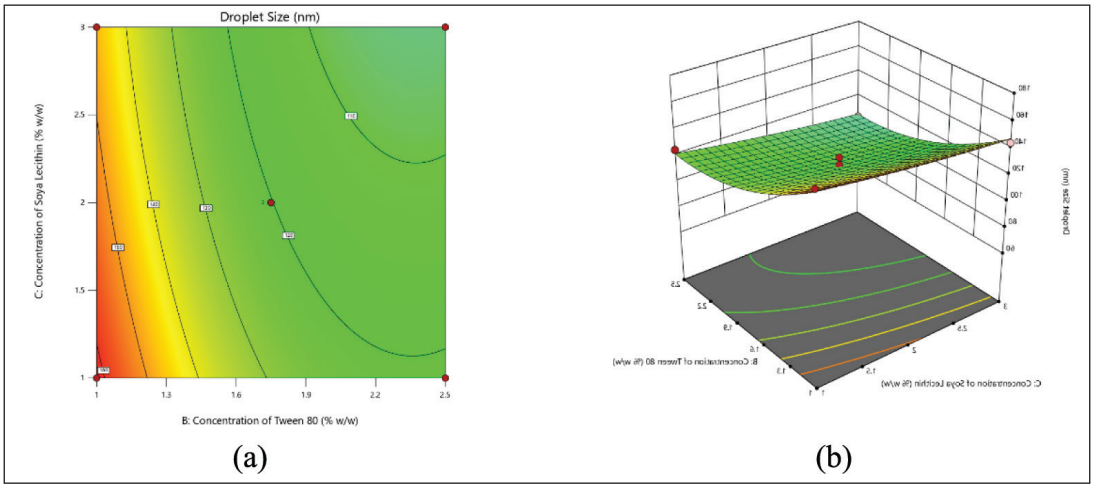


Figure 4. Effect of concentration of Tween 80 and concentration of soya lecithin on droplet size: (a) contour plot and (b) 3D surface plot.

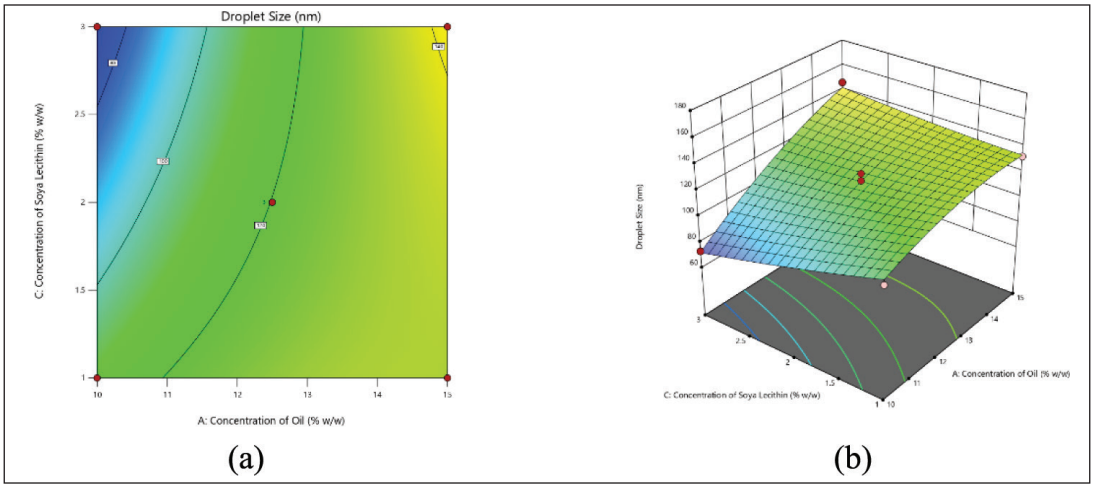


Figure 5. Effect of concentration of oil and concentration of soya lecithin on droplet size: (a) contour plot and (b) 3D surface plot.

Table 4. ANOVA of Box–Behnken design for transmittance.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value	
Model	15,318.19	9	1,702.02	743.75	<0.0001	**Significant**
A-Concentration of oil	79.44	1	79.44	34.72	0.0020	
B-Concentration of Tween 80	49.70	1	49.70	21.72	0.0055	
C-Concentration of soya lecithin	75.22	1	75.22	32.87	0.0023	
AB	12.46	1	12.46	5.45	0.0669	
AC	2,006.59	1	2,006.59	876.84	<0.0001	
BC	8,241.01	1	8,241.01	3,601.17	<0.0001	
A^2	237.37	1	237.37	103.73	0.0002	
B^2	349.89	1	349.89	152.90	<0.0001	
C^2	4,202.55	1	4,202.55	1,836.44	<0.0001	
Residual	11.44	5	2.29			
Lack of fit	3.86	3	1.29	0.3397	0.8039	**Not significant**
Pure error	7.58	2	3.79			
Cor total	15,329.63	14				

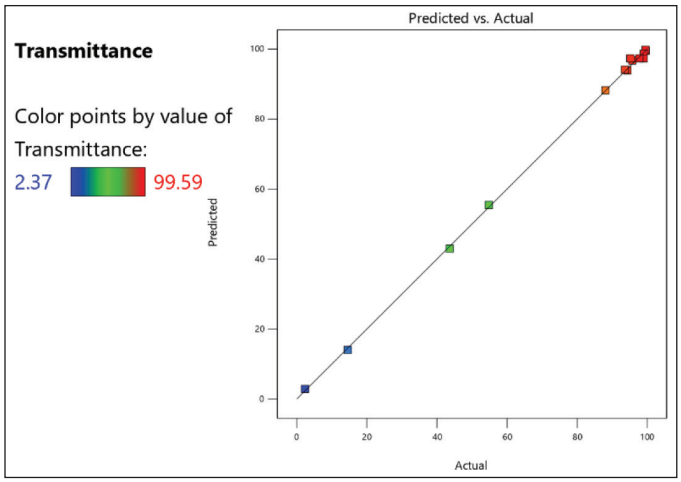


Figure 6. Graph between predicted and actual transmittance.

0.9568% or 95.68%, and a strongly connected adjusted R^2 value of 0.8660% or 86.60%. Figure 2 illustrates the actual and predicted response.

The following equation was obtained by analysis.

$$\text{Droplet size} = 120.26 + 22.8737 \times A + -20.5462 \times B + -7.75 \times C + 5.5575 \times AB + 12.075 \times AC + -0.46 \times BC + -7.03375 \times A^2 + 12.4162 \times B^2 + 1.48875 \times C^2$$

Coefficient values show that the concentration of oil had a positive effect on the droplet size. Oil concentration increases the droplet size of the nanoemulsion. Formulations 2, 8, and 9 have a higher droplet size (162.48, 163.95, and 145.93, respectively), due to the high concentration of oil and low concentration of surfactants. Where concentration of Tween 80 and soya lecithin had negative coefficient value which help to decrease the droplet size of nanoemulsion. Formulations 3, 7, and 12 have shown smaller droplet size (77.69, 72.94, and 103.46, respectively), due to the presence of a higher concentration

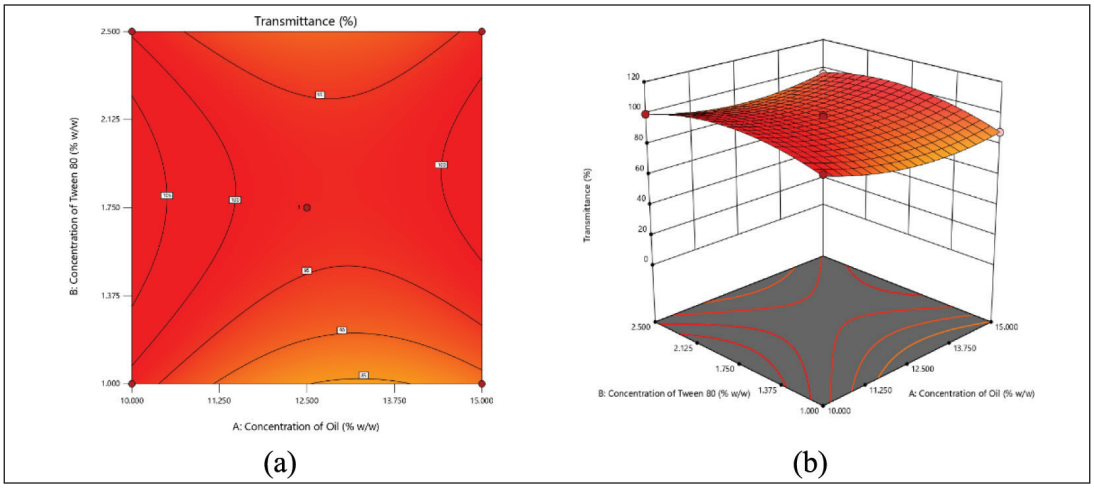


Figure 7. Effect of concentration of oil and concentration of Tween 80 on transmittance: (a) contour plot and (b) 3D surface plot.

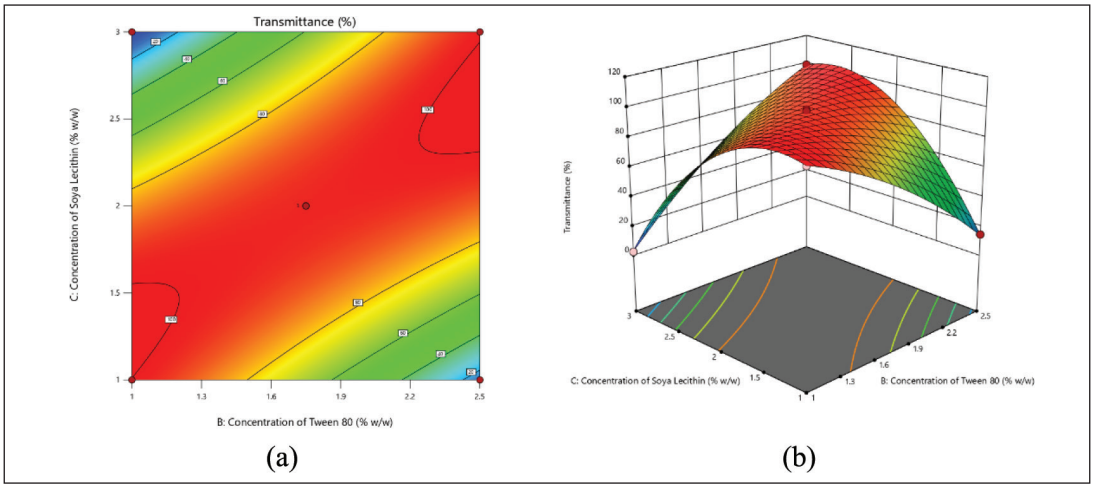


Figure 8. Effect of concentration of soya lecithin and concentration of Tween 80 on transmittance: (a) contour plot and (b) 3D surface plot.

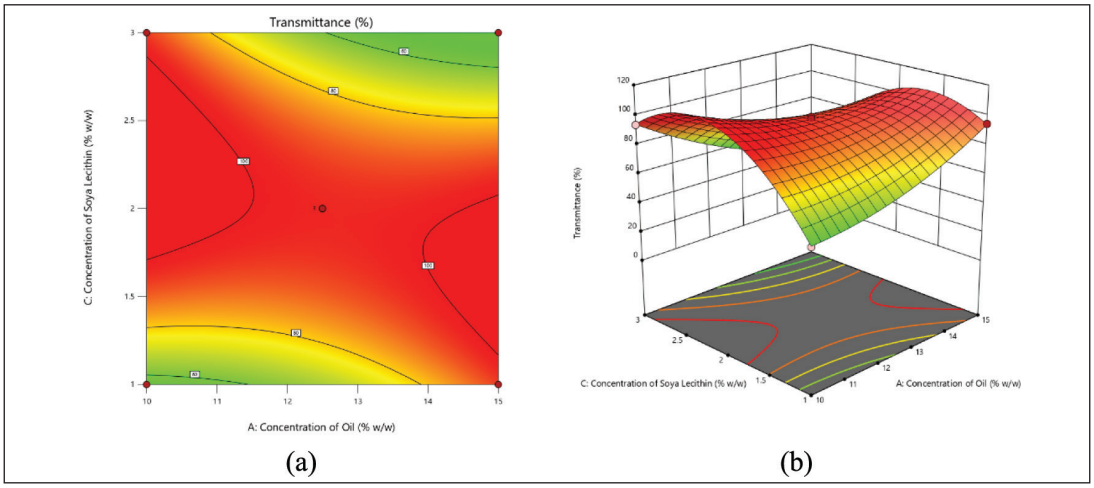


Figure 9. Effect of concentration of oil and concentration of soya lecithin on transmittance: (a) contour plot and (b) 3D surface plot.

of surfactant. Furthermore, the surfactant and cosurfactant play crucial roles in the condensation and expansion of the interfacial film, respectively. In our formulation, tween 80 was employed as the surfactant; being an ester of long-chain fatty acids, it facilitates the reduction of droplet size [18]. Previous studies also showed that droplet size increases with an increase in concentration of oil, while increasing concentration of surfactant and cosurfactant decreases the droplet size [42]. The response surface method generates a contour plot or 3D surface plot that indicates the relationship between two independent variables, specifically related to droplet size. Contour plot and 3D surface plot between the concentration of oil (A) and concentration of tween 80 (B), concentration of tween 80 (B) and concentration of soya lecithin, (C) and concentration of oil (A) and concentration of soya lecithin (C) are presented in Figures 3–5, respectively.

3.2.2. Effect of the independent variable on transmittance

Experimental results of transmittance were examined to determine the effect of the independent variable (Table 2). Transmittance ranges between 2.37 and 99.59, with an average of 78.41. After statistical analysis, ANOVA selects the quadratic model for the determination of significant value, lack of fit, and multiple correlation coefficients. The model had a significant *p*-value of 0.0001 and a non-significant lack of fit value of 0.3397 (Table 4). Based on this, the selected model significantly described the effect of the independent variable on the response. The obtained R^2 value in this study is 0.99983% or 99.93%. The chosen model had a predicted R^2 value of 0.9979% or 99.79%, and adjusted R^2 value of 0.9949% or 99.49%. The *F* value shows the model's fitness. A higher *f* value indicates that the independent variable shows a high effect on the response variable. Figure 6 illustrates the actual and predicted response. Figure 6 illustrates the actual and predicted response.

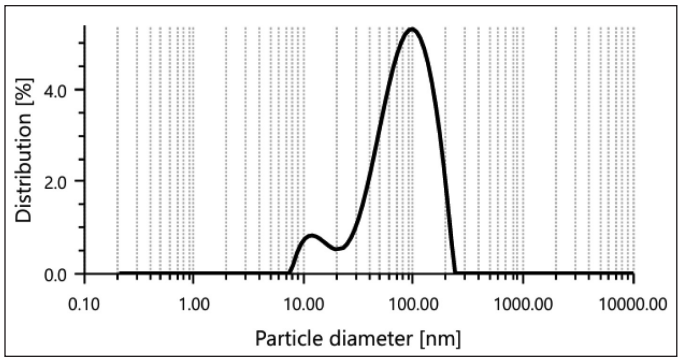


Figure 10. Droplet size of plant extract-loaded nanoemulsion.

The following equation was obtained by analysis.

$$\text{Transmittance} = 97.3167 + -3.15125 \times A + 2.4925 \times B + -3.06625 \times C + 1.765 \times AB + -22.3975 \times AC + 45.39 \times BC + 8.01792 \times A^2 + -9.73458 \times B^2 + -33.7371 \times C^2$$

Coefficient values show that the concentration of Tween 80 had a positive effect on the transmittance. Tween 80 increases the transmittance of the nanoemulsion. Formulations 3, 12, and 14 have a high concentration of Tween 80, which increases the transmittance (99.59, 98.99, and 98.99, respectively). This confirms that Tween 80 have a positive effect on transmittance. Where concentration of oil and soya lecithin had negative coefficient value which decrease the transmittance of nanoemulsion. Formulations 8, 10, and 11 have a high concentration of oil and cosurfactant, which decreases the transmittance (43.62, 14.54, and 2.37, respectively). The response surface method generates a contour plot or 3D surface plot that indicates the relationship between two independent variables, specifically related to droplet size. Contour plot and 3D surface plot between the concentration of oil (A) and concentration of Tween 80 (B), concentration of Tween 80 (B), concentration of soya lecithin (C), concentration of oil (A), and concentration of soya lecithin (C) are presented in Figures 7–9.

3.3. Optimization of nanoemulsion

The Design Expert 13 software was used for optimization of independent variables and response variables. The independent variables were assigned a range value, and constraints were applied to the response variable. The droplet size was set to the minimum value, and the transmittance was set to the maximum value. On the basis of constraints, 100 solutions were generated with desirability scores ranging from 0.769 to 1.000. The optimized formulation had a predicted concentration of oil (10.12% w/w), concentration of Tween 80 (1.83% w/w), and concentration of soya lecithin (2.93% w/w), with a droplet size and transmittance of 72.58 nm and 99.79%, respectively. The formulation was tested, showing significant closeness to the predicted response value and stability in evaluation tests. The optimized formulation shows the droplet size of 70.72 nm (Fig. 10), with 99.21% transmittance (Table 5).

3.4. Drug-excipient interaction

FTIR analysis was carried out to investigate the interaction between the plant extract and excipients of the nanoemulsion formulation. The FITR spectra of PCHE, FIHE, AJHE, oleic acid, olive oil, Tween 80, and soya lecithin are shown in Figure 11. The FTIR spectrum of oleic acid shows peak around 3,008, 2,923.05, 2,853.94, 1,708.5, 1,464.29,

Table 5. Predicted response value and experimental response value.

Solution 1 of 100 responses	Concentration of oil (% w/w)	Concentration of Tween 80 (% w/w)	Concentration of Soya lecithin (% w/w)	Droplet size (nm)	Transmittance (%)
Predicted response	10.12	1.83	2.93	72.58	99.79
Experimental response	10.12	1.83	2.93	70.72 ± 1.4	99.21 ± 0.6

Mean ± SD; n=3: Standard deviation

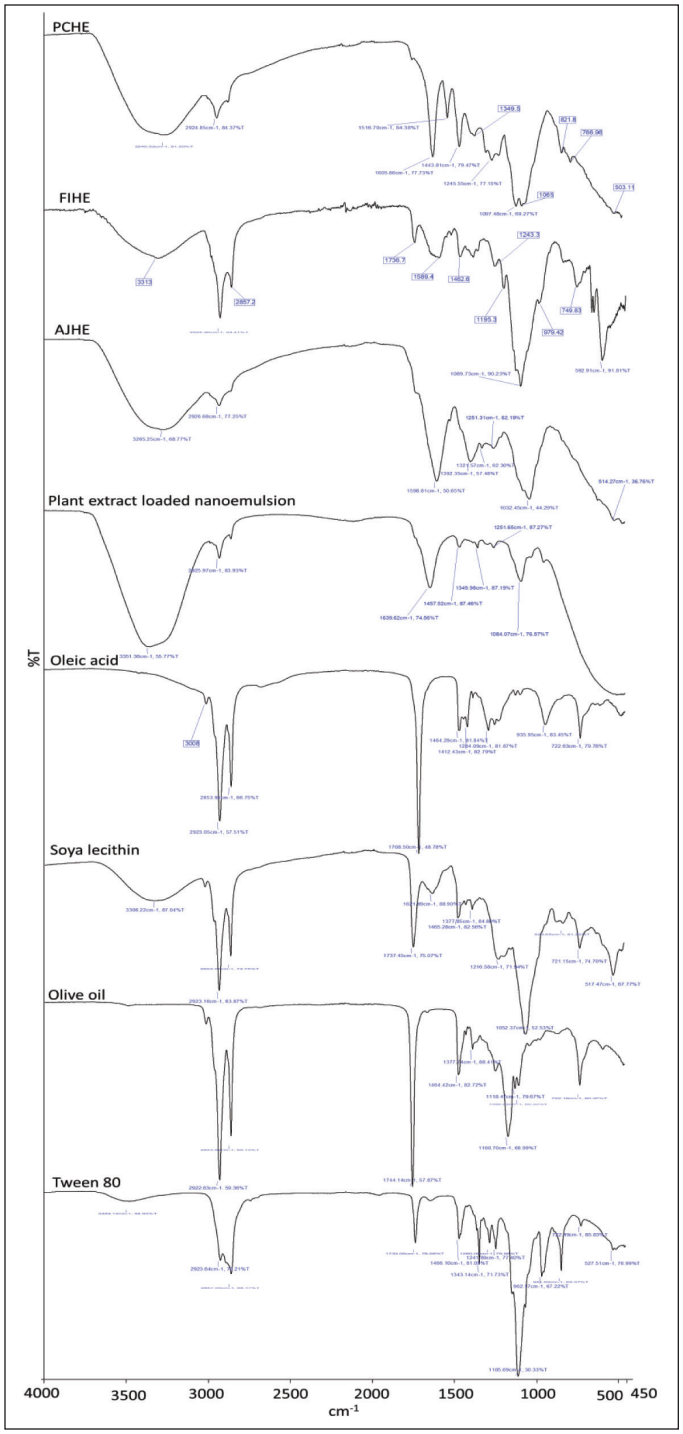


Figure 11. FTIR spectra of PCHE, FIHE, AJHE, plant extract-loaded nanoemulsion, oleic acid, soya lecithin, olive oil, and Tween 80.

1,412.43, 1,284.09, 935.95, and 722.63 cm^{-1}. These peaks show the presence of OH, C-H, CH_2, CH_3, C=O, COOH, and C=C-H functional groups in the oleic acid. FTIR spectra of olive oil show peaks around 2,922.83 cm^{-1} (CH), 2,853 cm^{-1} (CH_2, CH_3), 1,744.17 cm^{-1} (C=O), 1,377.74 cm^{-1} ($CH(CH_3)_2$), 1,160.70 cm^{-1} (C-O-C, C-N-,), and 1,118.47 cm^{-1} (C=S, COH). Tween 80 FTIR spectra show the major peaks around 3,484.12 cm^{-1} (OH), 2,923.64 cm^{-1}, 2,857.03 cm^{-1} (CH), 1,733.05 cm^{-1}

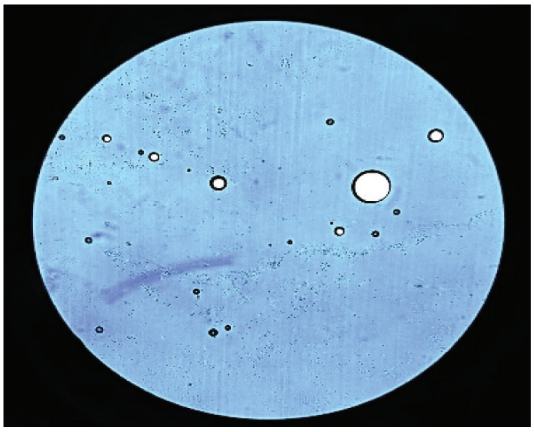


Figure 12. Optical microscopy of methylene blue-loaded nanoemulsion.

(C=O), 1,466.10 cm^{-1}, 1,343.14 cm^{-1} (CH_2, CH_3), and 962.17 cm^{-1} ($CH=CH_2$). Soya lecithin shows peaks around 3,306 cm^{-1} (OH), 2,923.16, 2,453 cm^{-1} (CH), 1,737.45 cm^{-1} (C=O), 1,621.86 cm^{-1} (C=C), 1,465.28 cm^{-1},1,377.85 cm^{-1} (CH_2, CH_3, $CH(CH_3)_2$), 1,216.58 cm^{-1} (C-O-C), and 823.65 cm^{-1} (C=C-H). The major peaks observed in FTIR spectra of plant extract-loaded nanoemulsion were found at around 3,351.36 cm^{-1} (OH, NH), 2,925.97 cm^{-1} (CH, COOH), 1,639.62 cm^{-1} (C=C, RONO, $RONO_2$), 1,457.52 cm^{-1} (CH_2, CH_3), 1,349.98 cm^{-1} ($C-NO_2$), 1,251.65 cm^{-1} (-C-N-, C-O-C), and 1,084.07 cm^{-1} (Si-O-Si, Si-O-C). The FTIR spectra of plant extract-loaded nanoemulsion have shown that the major functional groups present in plant extracts were incorporated in the plant extract-loaded nanoemulsion formulation. These spectra confirmed the absence of any interaction between the compounds of plant extracts, excipients, and nanoemulsion formulation [43,44].

3.5. Microscopic evaluation of the nanoemulsion

The microscopic examination of plant extract-loaded nanoemulsion was carried out to identify the nature of the dispersed phase and continuous phase, as shown in Figure 12. The image represented a dark blue, continuous background with colorless globules dispersed in it. This confirmed that nanoemulsions have an oil phase as the dispersed phase and an aqueous polar phase as the continuous phase.

3.6. Zeta potential and polydispersity index

The zeta potential of plant extract-loaded nanoemulsion was found to be −15.9 mV, as shown in Figure 13. Higher zeta potential values show that the nanoemulsion formulation was more stable. The polydispersity index determines the homogeneity or heterogeneity of particle size within the formulation. In the literature review, it is reported that polydispersity indices below 0.3 were considered as homogeneous nanoemulsions [45]. The polydispersity index of the plant extracts-loaded nanoemulsion was 0.259; it shows monodispersed stability of the nanoemulsion.

3.7. Thermodynamic stability study

Plant extract-loaded nanoemulsion was placed in centrifugation for 30 minutes and also stored at 4°C and 45°C temperatures for 24 hours to assess the thermodynamic stability

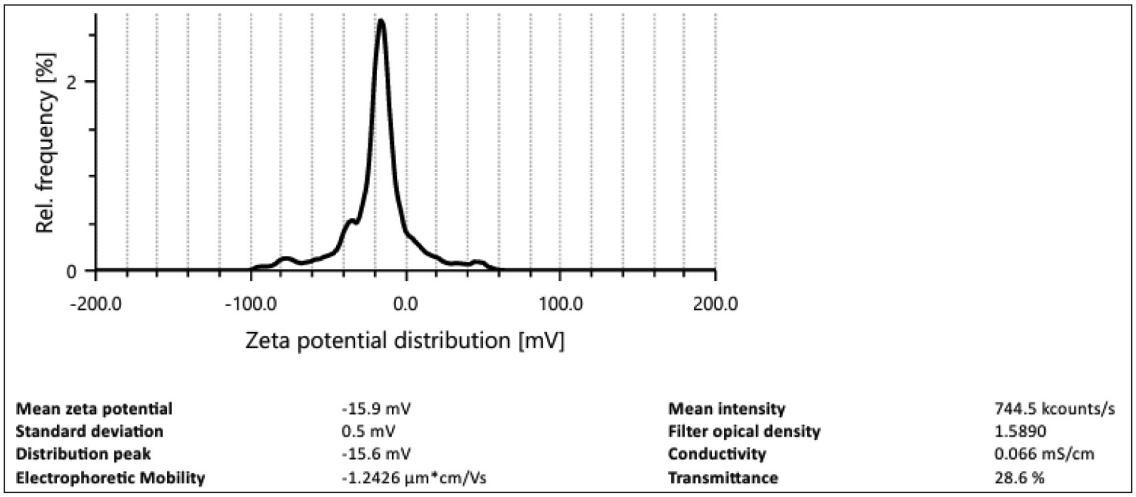


Figure 13. Zeta potential of plant extract-loaded nanoemulsion.

of the nanoemulsion. Optimized nanoemulsion does not show any type of phase separation and creaming. It shows that the formulation was thermodynamically stable.

3.8. Dilution test

A dilution test was carried out to check if any type of phase separation occurs in the formulation. After dilution, nanoemulsion with distilled water does not show any phase separation and remains stable. Thus, it confirms that the optimized nanoemulsion formulation was stable.

3.9. Organoleptic properties of nanoemulsion gel

The prepared nanoemulsion gel was transparent with a smooth appearance without any aggregations. The optimized formulation has shown no signs of phase separation or liquefaction. This effect may be attributed to the presence of non-ionic surfactants, which are recognized for their stability against variations in ionic strength and pH of the incorporated agents [18,46].

3.10. pH estimation

The pH of the nanoemulsion gel was suitable for topical use. The pH of the nanoemulsion gel was 5.75, indicating slightly acidic pH. Hence, it would be non-irritant and compatible with skin [47].

3.11. Spreadability of nanoemulsion gel

The spreadability test confirms the shear-thinning behavior of the nanoemulsion gel and its suitability for smooth application [48]. The spreadability value of gel was 0.546. Good spreadability allows for easier and more uniform application of the nanoemulsion gel on the skin, improving patient comfort and compliance [49].

3.12. Stability studies

A stability study helps to analyze the long-term storage of nanoemulsions without any degradation. Prepared plant extract-loaded nanoemulsion exhibited satisfactory stability, with no occurrence of phase separation or creaming. This instability does not occur due to the presence of an adequate amount of surfactant [46]. After 1 month of storage of nanoemulsion at different temperature ranges, a slight increase in the droplet size was observed, as shown in Figure 14. Throughout the study, there was no alteration in odor or color, and no microbial growth was observed, which may be due to the presence of ethanol in the plant extract [50]. Tween 80, a non-ionic surfactant, plays a crucial role in nanoemulsion formulation by reducing droplet size, improving stability, and enhancing encapsulation efficiency. It acts as an emulsifier, facilitating the dispersion of an oily phase in a water-based continuous phase [51]. Soy lecithin enhances the stability of nanoemulsions by acting as an effective emulsifier and surfactant, reducing droplet size and promoting a stable interface [52]. Both Tween 80 and soy lecithin, together as emulsifiers in nanoemulsions, can improve stability by reducing droplet size and preventing creaming or sedimentation. This helps to prevent oil droplets from coalescing and separating from the aqueous phase, thus enhancing the long-term stability of the nanoemulsion [21]. The oil phase shows the significant effects on the stability of nanoemulsion for long-term storage [53]. Previous studies also reported the influence of oil phase composition in stable nanoemulsion formulation [54].

3.13. *In vitro* anti-inflammatory activity

3.13.1. Protein denaturation assay

In the present study, the nanoemulsion of plant extract showed inhibition of protein denaturation, which validates its anti-inflammatory effects. Nanoemulsion of plant extracts showed a percentage inhibition of protein denaturation (65.82% ± 1.31%, 67.87% ± 1.35%, 71.86% ± 1.43%, and 78.98% ± 1.57%) at different concentrations (100, 200, 500, and 1000 µg/ml, respectively) in a concentration-dependent manner. Results of the percentage inhibition of protein denaturation are depicted in Figure 15. Nanoemulsion showed a high percentage of inhibition of protein denaturation as compared to the standard plant extract. The anti-inflammatory effect is probably due to a fair amount of flavonoid and phenolic contents measured in AJHE, FIHE, and PCHE, which act by inhibiting the inflammatory mediators [9,12,17,]. Thus, *P. cineraria*, *A. javanica*, and *F. indica* hydroalcoholic extract

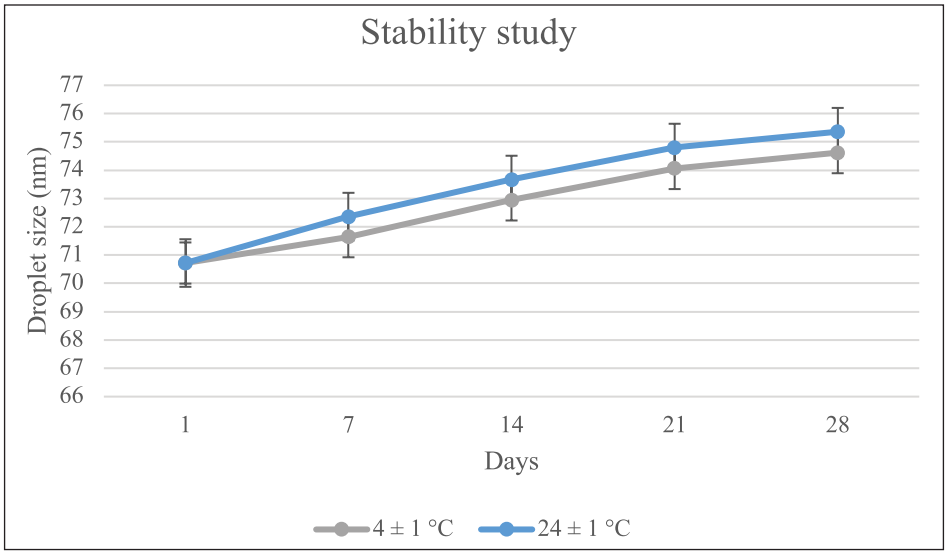


Figure 14. Change in droplet size of plant extract-loaded nanoemulsion.

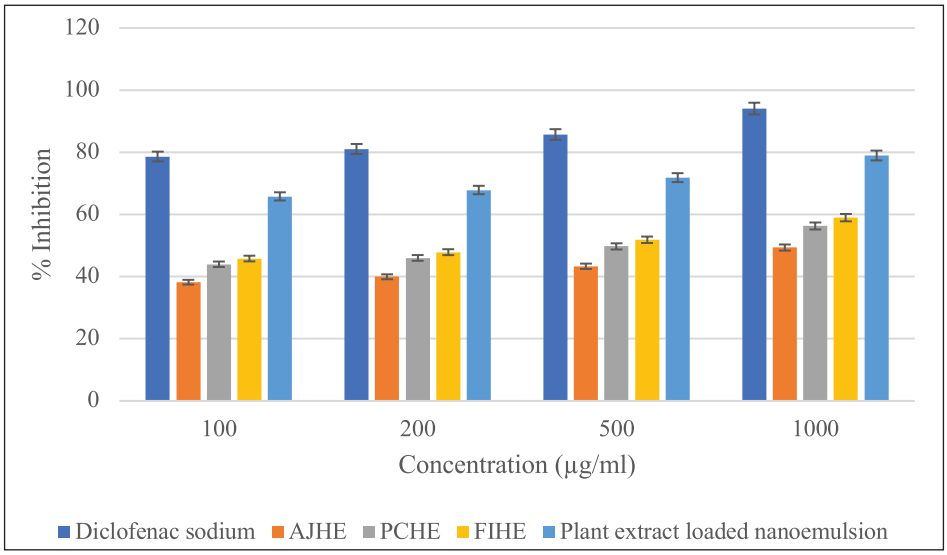


Figure 15. Percentage inhibition of protein denaturation.

loaded nanoemulsion could be a natural remarkable substitute remedy for the management of inflammatory disorders.

4. CONCLUSION

The optimum formulations of nanoemulsion containing natural extracts of (*P. cineraria*, *A. javanica*, and *F. indica*) were fabricated to enhance the solubility, bioactivity, and stability of plant bioactive compounds. The 0.2% w/w natural extract-loaded nanoemulsion with varying concentration of oil phase (oleic acid and olive oil), surfactants (Tween 80 and soya lecithin) was developed by using the ultrasonication method. A total of 15 runs were obtained by Box–Behnken design for nanoemulsion optimization. The optimized nanoemulsion represents oil phase 10.83 % w/w, surfactant 1.83% w/w, and cosurfactant 2.83% w/w with a minimum droplet size and maximum transmittance of 70.72 nm and 99.2%, respectively. Thus, it showed that experimental results were well in agreement with the predicted values. Similarly, developed nanoemulsion gel demonstrated significant spread ability, skin-friendly pH, including thermodynamic stability. Furthermore, nanoemulsion showed the potential for significant inhibition of protein denaturation, which validates its anti-inflammatory effect. The overall outcomes of the research will pave the way in the design of phytobioactive compounds incorporated into stable nanoemulsions with optimum independent variables. The primary objective of the present study was to formulate, optimize, characterize, and evaluate the *in vitro* anti-inflammatory activity of stable plant extract- loaded nanoemulsion system. However, for in-depth bioactivity analysis of nanoemulsion, in future research studies, phytobioactive compounds will be isolated from plant extracts and incorporated into the optimized nanoemulsion. Moreover, *in vitro* release studies, including *in vivo* anti-arthritic activity of nanoemulsion gel will be carried out for more comprehensive insights into the release dynamics and biological activity of traditionally

acclaimed plants. In conclusion, this study delineates that natural extracts loaded nanoemulsion gel might be investigated for effective topical delivery of phytobioactive compounds to treat arthritis and other inflammatory disorders.

5. ABBREVIATIONS

AJHE, *Aerva javanica* hydroalcoholic extract; ANOVA, analysis of variance; FIHE, *Fagonia indica* hydroalcoholic extract; FTIR, Fourier transform infrared; PCHE, *Prosopis cineraria* hydroalcoholic extract; RA, rheumatoid arthritis.

6. ACKNOWLEDGMENT

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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