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Response Surface Optimisation of a *Strobilanthes crispus*-Loaded Red Palm Oil Nanoemulsion with Enhanced Physicochemical Stability

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ABSTRACT

Plant-derived lipophilic bioactive compounds often show poor dispersion and instability in a water system; this limits their use in topical and pharmaceutical formulations. This study aimed to develop and optimise an oil-in-water nanoemulsion containing *Strobilanthes crispus* extract dissolved in red palm oil using Response Surface Methodology (RSM). A three-factor Box-Behnken design was applied to evaluate the effects of Tween® 80 concentration, glycerol concentration, and homogenisation pressure on droplet size, polydispersity index (PDI), and creaming index. The results show that the surfactant concentration was the most important factor affecting the droplet size and overall stability. The optimised nanoemulsion formulation comprised 5.06% (w/w) Tween® 80, 10.4% (w/w) glycerol, and a homogenisation pressure of 617.1 bar. Experimental validation produced a nanoemulsion with a droplet size of 151.8 ± 1.3 nm, PDI of 0.127 ± 0.021, and no visible creaming. The regression models showed strong predictive ability for droplet size ($R^2 = 0.984$) and creaming index ($R^2 = 0.999$), but moderate prediction for PDI ($R^2 = 0.7516$). Stability studies under different pH (2-6),

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temperature (40-60°C), ionic strength (0.5-2.5mM), and centrifugal conditions (1000-5000rpm), as well as four-month storage, showed that the nanoemulsion remained physically stable without phase separation. This stability is mainly attributed to steric stabilisation from the non-ionic surfactant. Overall, the study demonstrates that Response Surface Methodology is an effective tool for optimising nanoemulsion formulations for the delivery of lipophilic plant bioactives.

Keywords: Box-Behnken design, lipophilic bioactive, nanoemulsion, physicochemical stability, red palm oil, response surface methodology, *Strobilanthes crispus*

INTRODUCTION

Nanoemulsions are kinetically stable colloidal dispersions consisting of two immiscible liquids, usually stabilised by a surfactant, with droplet diameters typically in the range of 20-200nm. Their small droplet size gives nanoemulsions a larger surface area, which enhances the dispersion of hydrophobic compounds in aqueous systems and improves their bioavailability (Preeti et al., 2023). Oil-in-water (O/W) nanoemulsions are widely used in pharmaceutical and cosmetic applications as they allow lipophilic bioactives to be effectively incorporated into water-based formulations (Shakeel et al., 2021). Nanoemulsions also exhibit enhanced physical stability by minimising instability mechanisms such as creaming, flocculation, and coalescence, which contribute to improved shelf-life (Marhamati et al., 2021).

The development and stability of nanoemulsions are influenced by both formulation components and processing parameters. Surfactants reduce interfacial tension and stability of droplets, while co-solvents like glycerol affect the system's viscosity and density, influencing the droplet movement and stability. High-pressure homogenisations play a key role in reducing droplet size and ensuring a homogeneous dispersion (Zhou et al., 2022). Response Surface Methodology (RSM) is often used to evaluate the interaction between these variables and identify the optimal formulation conditions (Podder & Mukherjee, 2024).

Strobilanthes cripus (*S.crispus*) is a medicinal plant widely used in Southeast Asia and is known for its antioxidant, anti-inflammatory, and antiproliferative properties (Zhu et al., 2022). Studies have shown that *S.crispus* bioactive compounds can help regulate keratinocyte proliferation and reduce skin inflammation, which is useful for topical applications (Imam et al., 2023). Red palm oil can also be used for skin applications as it is rich in tocotrienols and carotenoids, which have strong antioxidant properties and help protect the skin from oxidative damage (Tan et al., 2021). Since these compounds are lipophilic, they are unlikely to dissolve in water-based formulations, so a suitable delivery system is required to ensure their stability and bioactivity.

In this study, an oil-in-water nanoemulsion containing red palm oil and *S.crispus* extract was developed and optimised using a Box–Behnken design within Response

Surface Methodology. The effects of formulation and processing variables on droplet size, polydispersity index, and physical stability were evaluated. The optimised nanoemulsion was then tested under various storage and stress conditions, and the results showed it remained stable over time. These findings highlight that nanoemulsion design can not only stabilise lipophilic bioactives but also enhance their delivery and efficacy as natural skin-protective ingredients.

MATERIAL AND METHODS

Preparation of Oil-in-Water Coarse Emulsion

An oil-in-water (O/W) nanoemulsion system was developed. The oil phase was red palm oil with *Strobilanthes crispus* extract, and the water phase contained Tween® 80 and glycerol.

The formulation included 20% (w/w) red palm oil (Nutrolein®, Golden Palm Oil) with 0.008% (w/w) *S. crispus* extract from TKC Herbal Nursery Sdn. Bhd. (Seremban, Malaysia). Tween® 80 (15% w/w; Fisher Scientific, Loughborough, UK) was used as the surfactant, and glycerol (30% w/w; USP grade, InterMed Sdn. Bhd., Kuala Lumpur, Malaysia) was added to adjust the thickness and density of the water phase. Citric acid (0.1% w/w; Shandong Ensign Industry Co. Ltd., China) was added to improve surfactant action at the oil-water interface, enhancing stability and reducing droplet aggregation, while also acting as a preservative. Deionised water was added to make 100% of the formulation.

For the coarse emulsion, the oil and water phases were first mixed and stirred together. The mixture was then pre-emulsified using a high-speed homogeniser (Silverson L4R, Buckinghamshire, UK) at 5000-8000 rpm for 5-10 minutes at room temperature until a uniform coarse emulsion was formed.

Optimisation of High-pressure Homogenisation Cycles

The coarse emulsion from the pre-emulsification step was further processed using a high-pressure homogeniser (Panda 2 K, Niro Soavi, Lübeck, Germany) to produce the nanoemulsion.

A preliminary study was carried out to study the effect of homogenisation cycles on nanoemulsion droplet size and stability. The coarse emulsion was homogenised at a constant pressure of 600 bar for up to eight cycles. Samples were collected after each cycle (1-8 cycles) and analysed for the droplet size, polydispersity index (PDI), and visual stability to determine the optimal number of cycles required for nanoemulsion formation. High-pressure homogenisation is effective in reducing droplet size and producing uniform nanoemulsions as it generates strong shear, cavitation, and turbulence forces during processing (Chandel et al., 2025; Rauber et al., 2025)

The emulsion temperature was monitored during homogenisation and kept below 50°C (±5°C) to minimise potential degradation of heat-sensitive bioactive compounds. All experiments were performed in triplicate.

Preparation of Nanoemulsion

After the preliminary study was conducted to determine optimum homogenisation conditions and parameter range, a Box-Behnken design was used to evaluate the effect of homogenisation pressure, surfactant concentration, and glycerol concentration. RSM was applied to optimise these variables and analyse their interactions (Kumar & Reji, 2023).

The coarse emulsion prepared under optimised pre-emulsification was then processed using a high-pressure homogeniser to produce the nanoemulsion. Homogenisation was run with the optimum number of cycles determined from a preliminary study, with the pressure range 500 to 700 bar based on the experimental design. The process helps reduce droplet size through strong shear, turbulence and cavitation force.

The nanoemulsion was allowed to equilibrate at room temperature (24.0 ± 1.0°C) before the physicochemical characteristics study to ensure stabilisation of the droplet system.

Optimisation of Nanoemulsion Formulation using Response Surface Methodology

The nanoemulsion formulation was optimised using a Box-Behnken design (BBD) under Response Surface Methodology (RSM) to evaluate the effects of surfactant concentration (Tween® 80, 5-15% w/w), glycerol concentration (10-30% w/w), and homogenisation pressure (500-700 bar). Each variable was studied at three coded levels (-1,0,+1), corresponding to actual value presented in Table 1. These ranges were selected based on preliminary screening and literature to ensure adequate interfacial stabilisation, appropriate viscosity, and effective droplet size reduction (Chong et al., 2022; Lüdtke et al., 2022).

The experimental matrix of the Box–Behnken design (BBD), including the combinations of surfactant concentration (X_1), glycerol concentration (X_2), and homogenisation pressure (X_3), is presented in Table 2.

The oil-to-surfactant (O/S) ratio is a key determinant of droplet formation and emulsion stability, as it governs the balance between dispersed phase volume and interfacial coverage by surfactants. In this study, the oil phase was fixed at 20% (w/w) based on preliminary optimisation, where this concentration provided sufficient solubilisation of *Strobilanthes crispus* extract while maintaining formulation stability. Lower oil content resulted in insufficient loading, whereas higher oil content reduced stability due to droplet aggregation. This range is also consistent with reported stable nanoemulsion systems (20-30% oil fraction) (Sarheed et al., 2020; Luu et al., 2025). Red palm oil was selected as the oil phase due to its high content of carotenoids and tocotrienols, which contribute to antioxidant activity and support topical applications (Chong et al., 2018).

The *Strobilanthes crispus* extract concentration (0.008% w/w) was selected based on preliminary studies demonstrating effective antioxidant and anti-inflammatory activity for topical application, while ensuring complete solubility and formulation stability. This is supported by Loke et al. (2019), who reported strong antioxidative activity of *S. crispus* extract at low concentrations.

Optimisation was therefore achieved by varying surfactant concentration, which indirectly adjusted the O/S ratio while evaluating the effects of glycerol concentration and homogenisation pressure. Droplet size (Y_1), polydispersity index (PDI, Y_2), and creaming index (Y_3) were used as indicators of nanoemulsion quality and stability (Mukherjee & Baruah, 2025). The BBD consisted of 15 experimental runs, and the data were fitted to a second-order polynomial model to determine optimal formulation conditions.

Table 1

Coded levels of independent variables used in response surface methodology (RSM)

Independent Variables	**Symbols**	**Coded levels**		
		-1	**0**	**1**
Surfactant concentration (%)	X_1	5	10	15
Glycerol concentration (%)	X_2	10	20	30
Homogenisation Pressure (bar)	X_3	500	600	700

Table 2

Matrix of the Box-Behnken design (BBD)

Run	**X_1: Surfactant Concentration (% w/w)**	**X_2: Glycerol Concentration (% w/w)**	**X_3: Homogenisation Pressure (bar)**
1	15	20	700
2	5	30	600
3	15	20	500
4	5	20	700
5	10	20	600
6	10	30	500
7	15	30	600
8	10	10	700
9	10	20	600
10	5	10	600
11	10	30	700
12	5	20	500
13	10	20	600
14	10	10	500
15	15	10	600

Stability Studies of Emulsion

Evaluation of Droplet Size and Polydispersity Index

The mean droplet size and polydispersity index (PDI) of both coarse emulsion and nanoemulsion were measured using dynamic light scattering (DLS) with Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK). The sample was diluted 10-fold with deionised water to minimise multiple scattering effects and to prevent the interference from high particle concentration, which can affect the accuracy of DLS measurement (Rodriguez-Loya et al., 2023). Data collection and analysis were carried out using Malvern Zetasizer Software version 7.11.

Evaluation of Creaming Index

The coarse emulsion and nanoemulsion were monitored for the formation of a creaming layer to evaluate stability, following the method described by Mohammed et al. (2020). After 24 hours of incubation at room temperature, the height of the creamed layer (HL) and the total height of the emulsion (HE) were measured. The creaming index was then calculated using the formulation Equation 1:

$$CI = \frac{HL}{HE} \times 100 \qquad [1]$$

Short-term Stability Studies of Nanoemulsion

Short-term stability refers to accelerated stability studies in which different environmental stresses are applied to the sample, following the guidelines of the International Conference on Harmonisation (ICH). Freshly prepared nanoemulsion samples of 5mL were used for testing for different experimental conditions, which are summarised in Table 3. All samples were then incubated at room temperature (25°C) and stored for 48 hours, after which droplet size, PDI, and creaming index measurements were taken.

Long-term Stability Studies of Nanoemulsion

For long-term stability studies, 10 mL of newly prepared nanoemulsion was aliquoted into 15 mL polypropene centrifuge tubes and stored in the dark at 5°C, 25°C, and 40°C for four months.

Table 3
Experimental conditions for accelerated stability testing for nanoemulsion

Parameter	Conditions
pH adjustment	Samples were prepared with varying pH ranging from 2 to 6 using 1 N hydrochloric acid and 1 N sodium hydroxide solutions.
Heat treatment	Samples were incubated at temperatures ranging from 40°C to 60°C for 30 minutes in a water bath.
Ionic Strength	Samples were prepared with varying ionic strengths, ranging from 0.5 mM to 2 mM, by adding a 5 mM sodium chloride solution.
Centrifugation	Samples were centrifuged at speeds from 1000 to 5000 rpm for 30 minutes at room temperature.

Droplet size, PDI, and creaming index were measured at predetermined time points of 0, 2, 3, and 4 months.

Statistical Analysis

Experimental design and response surface methodology (RSM) analysis were performed using Design Expert version 13 (Stat-Ease, Inc., Minneapolis, Minnesota, USA). Measurements were carried out in triplicate and represented as mean ± standard deviation. One-way ANOVA was performed using Minitab Statistical Software version 21.2 (Minitab, LLC, Pennsylvania, USA), and Tukey's post hoc test was subsequently applied. Statistical significance was kept at $P < 0.05$ with a 95% confidence interval.

RESULTS AND DISCUSSION

Influence of Homogenisation Speed and Time on Coarse Emulsion Formation

High-speed homogenisation plays a critical role in producing a stable coarse emulsion by reducing droplet size, enhancing uniformity, and improving overall stability. Increasing the speed intensifies shear forces, resulting in smaller droplet sizes due to more efficient disruption of oil droplets within the aqueous phase. As shown in Figure 1, the droplet size decreased significantly as the speed increased. Additionally, extending homogenisation time from 5 to 10 minutes further reduced droplet size, indicating that prolonged shear allows more complete droplet breakup.

The result for the polydispersity index (PDI) shows that the higher homogenisation speeds generally produce lower PDI, as summarised in Table 4. This also indicates that the emulsion is characterised by a narrower droplet size distribution and greater uniformity. However, at the speed of 8000 rpm for 10 minutes, a slight and unexpected increase in PDI. This result may be explained by the droplet’s recoalescence due to excessive shear energy, which has a similar observation reported by Xu et al. (2018). As a result, a homogenising speed of 8000 rpm for 5 minutes was determined to be optimal for coarse emulsion conditions, producing fine droplets while minimising potential degradation of heat-sensitive bioactives, such as the phenolic compound in *S.crispus* extract (Chua et al., 2019).

Effect of High-pressure Homogenisation Cycle on Nanoemulsion Properties

High-pressure homogenisation affected the nanoemulsion properties, especially droplet size, polydispersity index (PDI), and physical stability. Increasing the number of homogenisation cycles reduced droplet size through shear, turbulence, and cavitation forces (Singh et al., 2017). Too many cycles, however, can cause overprocessing and lead to droplet aggregation because of more frequent collisions (Zhou et al., 2023). Therefore, it is important to choose the right number of cycles to balance size reduction and stability.

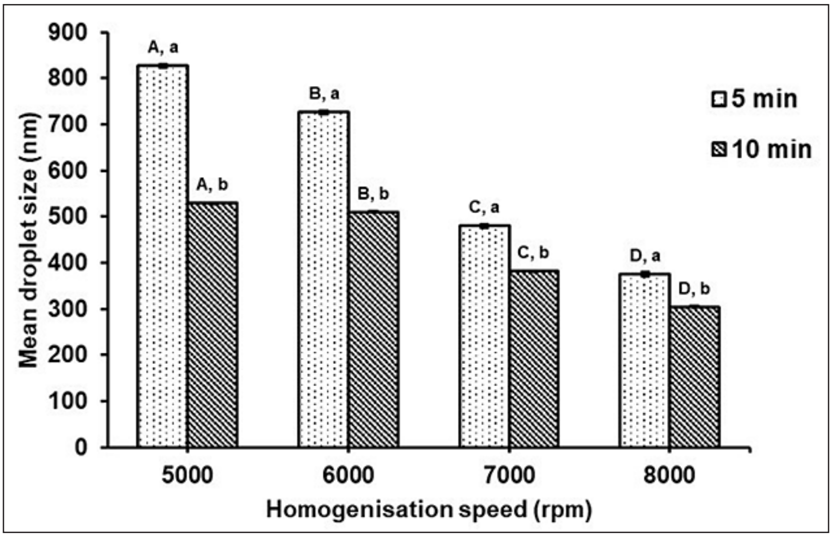

Figure 1. Effect of homogenisation speed and time on the mean droplet size of coarse emulsion
Note. Different uppercase letters indicate significant differences at the same homogenisation speed, whereas different lowercase letters indicate significant differences at the same homogenisation time

Table 4
Effect of homogenisation speed and time on the polydispersity index (PDI) of the coarse emulsion

Homogenisation Speed (rpm)	**Homogenisation Time (min)**	
	5	**10**
5000	0.440 ± 0.015A, a	0.452 ± 0.009A, a
6000	0.496 ± 0.006B, a	0.459 ± 0.008A, b
7000	0.429 ± 0.001A, a	0.421 ± 0.001A, b
8000	0.303 ± 0.017C, a	0.345 ± 0.015B, a

Note. Different uppercase superscript letters in the same column and lowercase superscript letters in the same row denote statistical significance ($P < 0.05$)

The mean droplet size decreased from 197.9 nm to 128.1 nm after eight cycles, showing effective droplet dispersion (Figure 2). This matches previous studies, where more cycles improved droplet break-up due to stronger shear and cavitation (Preiss et al., 2021). The PDI and creaming index at different homogenisation cycle are shown in Table 5. The PDI decreased with increasing cycles, showing better size uniformity (Santos et al., 2022). Samples processed with 1-6 cycles showed visible creaming, which indicates poor physical stability, likely caused by density differences (Chow et al., 2024). From cycle 7 onwards, the creaming index was 0.0, showing that the nanoemulsion was physically stable (Galvão et al., 2018).

The optimum number of cycles was chosen based on droplet size in the nanoscale range (100-200 nm), PDI below 0.2, and no creaming. The temperature was kept below 50°C (±5°C) to prevent heat damage. High temperatures can cause surfactant dehydration, destabilise the interface, and degrade heat-sensitive bioactive compounds (Iacob-Tudose et al., 2021). Cycles 7 and 8 both produced stable nanoemulsions. Cycle 7 was chosen as the best condition because it achieved the desired results with less processing. Extra cycles may slightly improve droplet dispersion but can also increase heat exposure (Zhou et al., 2023). Cycle 7 provides efficient emulsification while reducing unnecessary processing. These conditions were used in the following RSM optimisation.

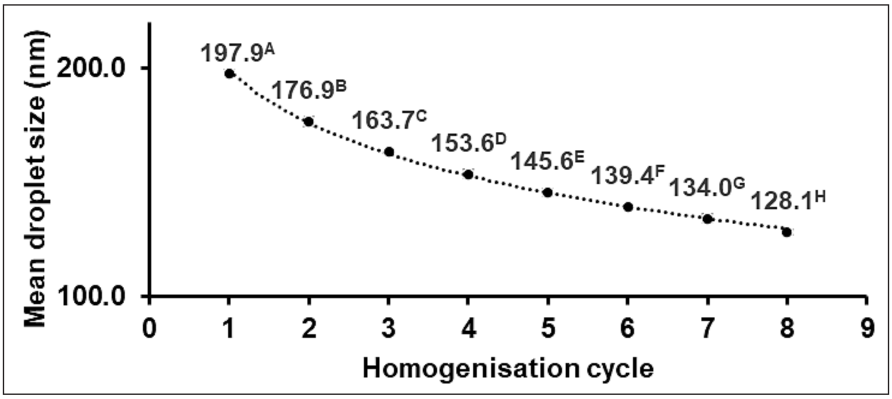

Figure 2. Mean droplet size of nanoemulsion at different high-pressure homogenisation cycles

Table 5
Polydispersity index and creaming index of nanoemulsion processed at different high-pressure homogenisation cycles

Homogenisation Cycle	PDI	Creaming Index
1	0.187 ± 0.009^{B}	42.42
2	0.147 ± 0.009^{A}	30.30
3	0.156 ± 0.017^{AB}	24.24
4	0.150 ± 0.005^{AB}	15.15
5	0.147 ± 0.007^{A}	9.09
6	0.153 ± 0.005^{AB}	6.06
7	0.135 ± 0.005^{A}	0.00
8	0.143 ± 0.005^{A}	0.00

Note. Superscript letters denote significant differences within columns ($p < 0.05$)

Optimisation of Nanoemulsion using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is a statistical method used to model and optimise processes with multiple variables and responses (Bakhaidar et al., 2022). In this study, a Box–Behnken design (BBD) was used to examine the effects of surfactant concentration, glycerol concentration, and homogenisation pressure on nanoemulsion properties. A total of 15 experimental runs were carried out to explore the formulation efficiently.

Figures 3, 4, and 5 show predicted versus actual response plots. The data points are closely clustered along the diagonal, which indicates good agreement between predicted and experimental values and confirms that the models are reliable. The normal probability plot for PDI showed a slight deviation from linearity, suggesting higher variability for this response. This may be due to the sensitivity of PDI to small changes in droplet size and processing conditions.

The coefficients of determination (R^2 and adjusted R^2) in Tables 6 to 8 also support good model fitting for droplet size and creaming index. Analysis of variance (ANOVA) showed that the quadratic models were statistically significant ($p < 0.05$), and the high F-values indicate strong effects of the formulation variables on the responses (Pongsumpun et al., 2020). The regression equations in Table 6,7 and 8 were used to predict the optimal formulation conditions for the nanoemulsion.

The regression equation for droplet size, expressed in terms of the actual factors, is as follows in Equation 2:

$$Y_1 = 216.88571 - 0.209571X_1 + 0.047750X_2 - 0.038875X_3 - 0.004820X_1X_2 - 0.002170X_1X_3 + 0.013086X_1^2 \quad [2]$$

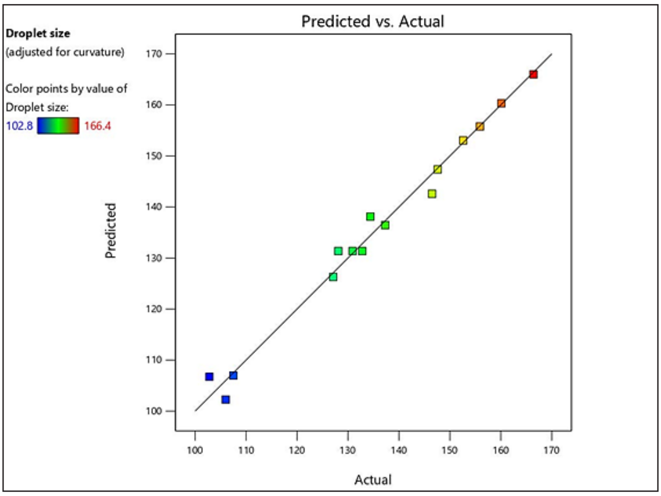

Figure 3. Predicted vs actual plot for droplet size of nanoemulsion

Table 6
ANOVA summaries and regression coefficients for droplet size, PDI and creaming index responses (Y_1)

Variable	**Droplet Size (Y_1)**		
	Mean Square	**F-value**	**P-value**
Intercept	890.54	96.72	<0.0001
Main effect			
X_1	2346.13	254.80	<0.0001
X_2	746.91	81.12	<0.0001
X_3	1737.55	188.71	<0.0001
Interaction effect			
X_1X_2	145.20	15.77	0.0041
X_1X_3	117.72	12.79	0.0072
Quadratic effect			
X_1^2	249.72	27.12	0.0008
Lack of fit	10.41	1.86	0.3897
R^2		0.9864	
Adjusted R^2		0.9762	

Note. X_1 = surfactant concentration (% w/w); X_2 = glycerol concentration (% w/w); X_3 = homogenisation pressure (bar)

The regression equation for the polydispersity index, expressed in terms of the actual factors, is as follows in Equation 3:

$$Y_2 = 0.115167 + 0.000535X_1 - 0.000155X_2 + 0.000024X_3 \qquad [3]$$

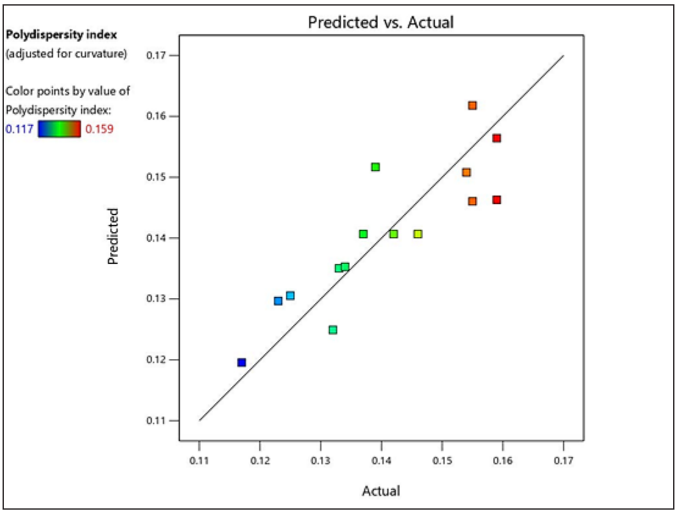

Figure 4. Predicted vs actual plot for polydispersity index of nanoemulsion

Table 7
ANOVA of the regression coefficient of the fitted quadratic equations for the polydispersity index (Y_2)

Variable	Polydispersity index (Y_2)		
	Mean Square	**F-value**	**P-value**
Intercept	0.0007	11.10	0.0012
Main effect			
X_1	0.0014	24.35	0.0004
X_2	0.0005	8.17	0.0155
X_3	0.0000	0.7677	0.3997
Lack of fit	0.0001	3.31	0.2535
R^2		0.7516	
Adjusted R^2		0.6839	

Note. X_1 = surfactant concentration (% w/w); X_2 = glycerol concentration (% w/w); X_3 = homogenisation pressure (bar)

The regression equation for the creaming index, expressed in terms of the actual factors, is as follows in Equation 4:

$$Y_3 = 87.91500 + 1.07710X_1 - 0.482975X_2 - 0.265213X_3 - 0.002904X_1X_2 - 0.001598X_1X_3 + 0.000714X_2X_3 + 0.003246X_1^2 + 0.000640X_2^2 + 0.000197X_3^2 \quad [4]$$

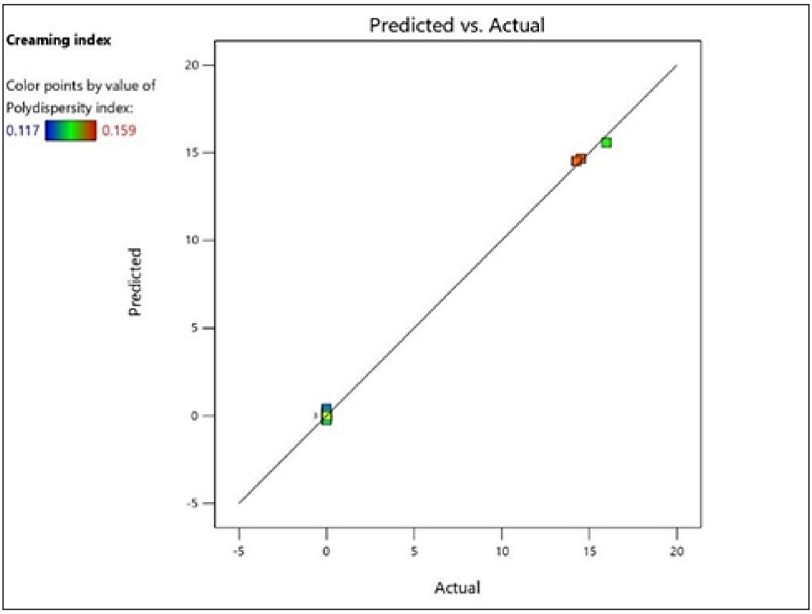

Figure 5. Predicted vs actual plot for creaming index

Table 8

ANOVA of the regression coefficient of the fitted quadratic equations for creaming index (Y_3)

Variable	**Creaming Index (Y_3)**		
	Mean Square	**F-value**	**P-value**
Intercept	59.51	465.06	<0.0001
Main effect			
X_1	116.25	908.77	<0.0001
X_2	103.61	809.72	<0.0001
X_3	114.36	893.93	<0.0001
Interaction effect			
X_1X_2	52.71	411.92	<0.0001
X_1X_3	63.84	498.93	<0.0001
X_2X_3	50.91	397.86	<0.0001
Quadratic effect			
X_1^2	15.20	1118.77	0.0001
X_2^2	9.47	73.99	0.0004
X_3^2	14.27	111.56	0.0001
Lack of fit	0.2133		
R^2		0.9988	
Adjusted R^2		0.9967	

Note. X_1 = surfactant concentration (% w/w); X_2 = glycerol concentration (% w/w); X_3 = homogenisation pressure (bar)

Influence of Independent Variables on Droplet Size

The results in Table 6 show that the model explained 98.64% of the variation in droplet size ($R^2 = 0.9864$; adjusted $R^2 = 0.9762$), indicating strong predictive ability and minimal overfitting. All main factors (X_1, X_2, and X_3) significantly affected droplet size ($p < 0.05$), with surfactant concentration having the strongest effect, followed by homogenisation pressure and glycerol concentration. This highlights that stabilising the oil-water interface is crucial for controlling droplet breakup and preventing recoalescence during high-pressure homogenisation. The optimised droplet size of 151.8 nm is smaller than many plant-based nanoemulsions, showing efficient droplet disruption and stabilisation under the selected conditions (Gupta et al., 2016; Singh et al., 2017).

It should be noted that droplet size measurements were performed under diluted conditions to ensure accuracy in dynamic light scattering (DLS) analysis. Although dilution may influence inter-droplet interactions, the same dilution protocol was consistently applied to all samples. Therefore, the results remain reliable for comparative evaluation of formulation and processing effects.

A significant interaction between surfactant concentration and homogenisation pressure (X_1X_3, $p < 0.05$) suggests that droplet size reduction depends on both interfacial coverage and mechanical energy. At low surfactant levels, increasing pressure alone does not reduce droplet size effectively because the interface is not fully stabilised, which can lead to droplet merging. At higher surfactant concentrations, there is enough coverage for the applied mechanical energy to break droplets into smaller, stable sizes. The quadratic effect of surfactant concentration (X_1^2) also indicates that there is an optimal surfactant level, beyond which further increases do not significantly reduce droplet size.

The response surface plot in Figure 6a-c illustrates the combined effect of surfactant concentration, glycerol concentration and homogenisation pressure on droplet size. Increasing surfactant concentration reduces droplet size by lowering interfacial tension and stabilising the droplets, thereby preventing coalescence. Similarly, increasing homogenisation pressure decreases droplet size. This explains that higher homogenisation pressure give stronger shear, turbulence, and cavitation forces break droplets more effectively (Gawin-Mikołajewicz et al., 2023; Kaur et al., 2024; Zhou et al., 2023). However, excessive mechanical energy does not always improve droplet size and may lead to temporary aggregation due to more frequent droplet collisions.

In contrast, glycerol concentration has a comparatively small effect on droplet size and does not show a significant interaction with homogenisation pressure ($p > 0.05$). This suggests that glycerol mainly affects the viscosity and density of the water phase, rather than directly contributing to droplets disruption. Higher viscosity can slow droplet movement and reduce creaming, but it has a limited effect on droplet formation during homogenisation (Gupta et al., 2016). This explains why glycerol had a smaller impact compared to surfactant and pressure.

The lack-of-fit test was not significant ($p > 0.05$), confirming that the model accurately represents the data within the studied range. Overall, these results show that successful nanoemulsion formation requires a balance between interfacial stabilisation and mechanical energy, with surfactant concentration being the most critical factor for achieving small, uniform droplets.

Influence of Independent Variables on Polydispersity Index (PDI)

The analysis in Table 7 shows that surfactant concentration (X_1) and glycerol concentration (X_2) significantly affected droplet size distribution ($p < 0.05$). Surfactant had the strongest effect ($F = 24.35$, $p = 0.0004$), followed by glycerol ($F = 8.17$, $p = 0.0155$). Homogenisation pressure (X_3) was not significant ($p = 0.3997$), suggesting that mechanical energy has less influence on droplet uniformity than surfactant coverage and the properties of the continuous phase (Singh et al., 2017). The lack-of-fit test was not significant ($p = 0.2535$), indicating the model fits the data well.

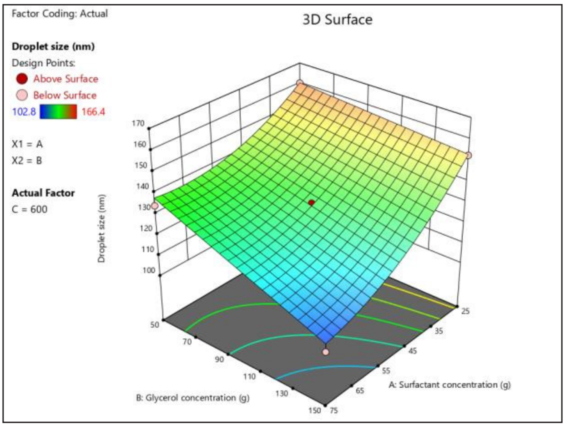

Figure 6a. Response surface and contour plots illustrating the interaction between surfactant concentration and glycerol concentration on the droplet size of the nanoemulsion

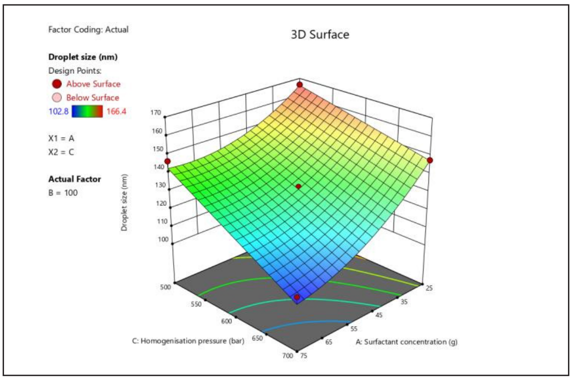

Figure 6b. Response surface and contour plots illustrating the interaction between surfactant concentration and homogenisation pressure on the droplet size of the nanoemulsion

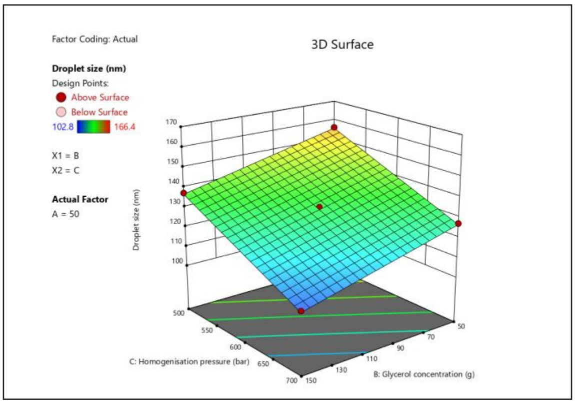

Figure 6c. Response surface and contour plots illustrating the interaction between glycerol concentration and homogenisation pressure on the droplet size of the nanoemulsion

The model's R^2 (0.7516) and adjusted R^2 (0.6839) show moderate predictability. This is lower than for droplet size because PDI is more sensitive to small changes in droplet distribution and processing conditions. PDI reflects the spread of droplet sizes, so minor variations in homogenisation efficiency, local concentration differences, or temporary droplet merging can affect it (Kaur et al., 2024). Other factors not included in the model, such as slight temperature changes or interfacial dynamics, may also contribute to variability (Zhou et al., 2023). Additionally, small differences in shear during homogenisation and the sensitivity of DLS to a few larger droplets can increase variation.

The response surface plots in Figure 7a–b illustrate the combined effects of surfactant concentration, glycerol concentration, and homogenisation pressure on the polydispersity index (PDI). Lower PDI values indicate more uniform droplets. In Figure 7a, increasing surfactant concentration reduced PDI by improving interfacial coverage and limiting droplet coalescence (Singh et al., 2017). Glycerol concentration shows a comparatively small effect, with a slight reduction in PDI at higher levels, likely due to increased viscosity of the continuous phase, which limits droplet movement and reduces aggregation (Gupta et al., 2016).

In Figure 7b, increasing both surfactant concentration and homogenisation pressure further decreases PDI, as stronger mechanical forces promote more efficient droplet breakup. No significant interactions were observed between glycerol concentration and homogenisation pressure ($p > 0.05$), suggesting that each factor mainly affects PDI independently.

Future studies could include additional variables, such as temperature control or surfactant combinations, to improve model reliability for predicting PDI.

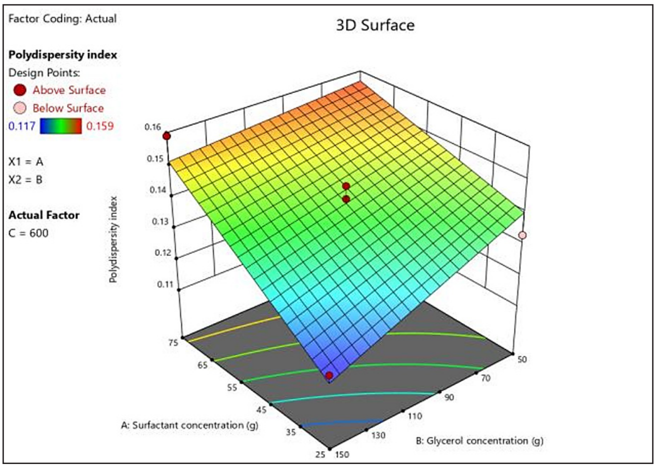

Figure 7a. Response surface and contour plots illustrating the interaction between surfactant concentration and glycerol concentration on the PDI of the nanoemulsion

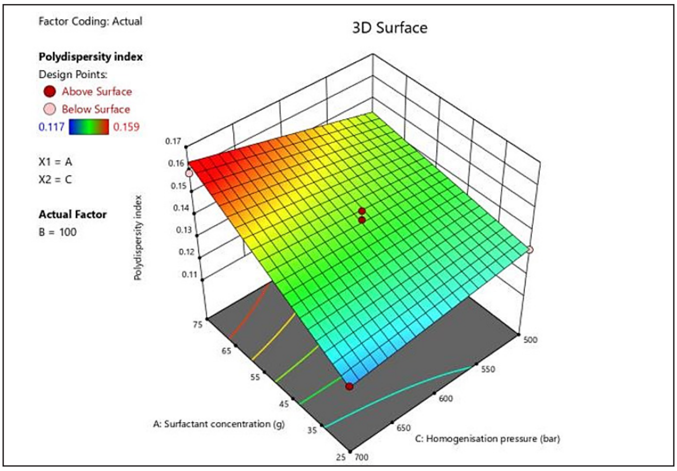

Figure 7b. Response surface and contour plots illustrating the interaction between surfactant concentration and homogenisation pressure on the PDI of the nanoemulsion

Influence of Independent Variables on Creaming Index

Table 8 shows the ANOVA results for the creaming index (Y_3). All three formulation variables, surfactant concentration (X_1), glycerol concentration (X_2), and homogenisation pressure (X_3), significantly affected nanoemulsion stability ($P < 0.0001$). The high F-values indicate that these factors strongly reduce creaming. All interaction terms (X_1X_2, X_1X_3, and X_2X_3) were also significant ($P < 0.0001$), showing that the combination of formulation and processing parameters plays an important role in stabilising the emulsion.

The quadratic effect of surfactant concentration (X_1^2) was especially strong ($F = 1118.77$, $P = 0.0001$), indicating a non-linear relationship between surfactant level and creaming. The model fit the data very well, with $R^2 = 0.9988$ and adjusted $R^2 = 0.9967$, and the lack-of-fit test was not significant ($P > 0.05$), confirming that the model reliably represents the experimental results. Overall, these results show that the optimised nanoemulsion is highly resistant to creaming and physically stable.

Figure 8 shows the response surface plots for the creaming index. Figure 8a demonstrates the interaction between surfactant and glycerol concentrations. Minimal creaming was observed when both variables were within an optimal range. Too much surfactant, especially at low glycerol levels, increased creaming, likely due to changes in interfacial structure or higher viscosity that limited droplet mobility. This highlights the need to balance surfactant and continuous phase modifiers to maintain stability.

Figure 8b shows the interaction between homogenisation pressure and surfactant concentration. At low surfactant levels, higher pressure improved stability by reducing droplet size and creaming. At high surfactant levels, low pressure was not enough to reduce droplet size, resulting in more creaming. This shows that mechanical energy must work together with surfactant coverage to achieve optimal nanoemulsion formation.

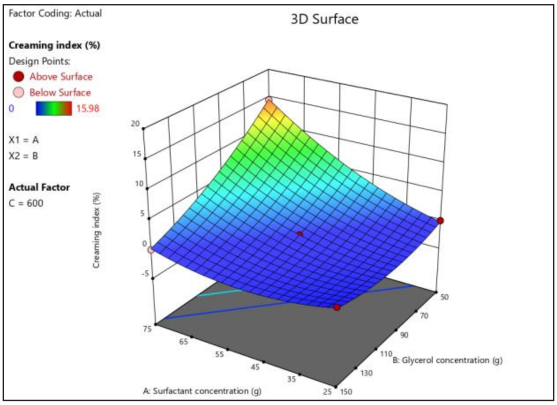

Figure 8a. Response surface and contour plots illustrating the interaction between surfactant concentration and glycerol concentration on the droplet size of the nanoemulsion

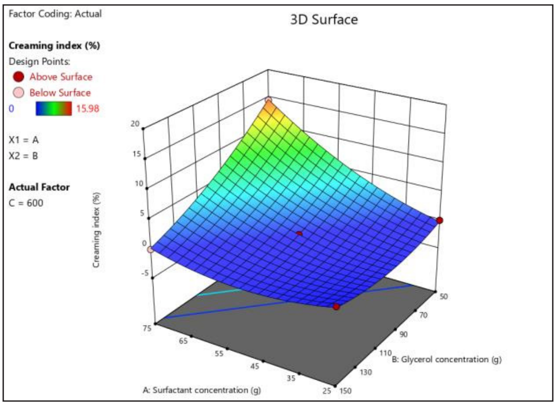

Figure 8b. Response surface and contour plots illustrating the interaction between surfactant concentration and homogenisation pressure on the droplet size of the nanoemulsion

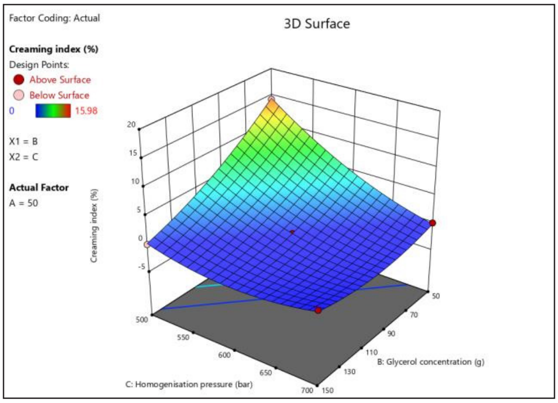

Figure 8c. Response surface and contour plots illustrating the interaction between glycerol concentration and homogenisation pressure on the droplet size of the nanoemulsion

Figure 8c shows that increasing glycerol concentration and homogenisation pressure decreased the creaming index, improving stability. This is mainly due to increased viscosity and density of the water phase, which slows droplet movement and reduces gravitational separation. This agrees with previous studies showing that higher continuous phase viscosity improves emulsion stability (Gupta et al., 2016; Singh et al., 2017). Figure 8a response surface and contour plots illustrating the interaction between surfactant concentration and glycerol concentration on the droplet size of the nanoemulsion

Optimisation Formulation of Oil-in-Water Nanoemulsion

The optimisation process aimed to develop a stable and cost-effective nanoemulsion with minimal surfactant and glycerol concentrations and homogenisation pressure while achieving a small droplet size, low polydispersity index (PDI), and absence of creaming. Based on model prediction, the optimum formulation consisted of 5.06% (w/w) surfactant and 10.4% (w/w) glycerol in the aqueous phase, with a homogenisation pressure of 617.1 bar. Under these conditions, the model predicted a droplet size of 158.2 nm, a PDI of 0.135, and a creaming index of 0.0%.

Experimental validation showed close agreement with the predicted values, producing a nanoemulsion with a droplet size of 151.8 ± 1.3 nm, a PDI of 0.127 ± 0.021, and no observable creaming. The strong correlation between predicted and experimental results confirms the accuracy and reliability of the response surface optimisation model. This agreement indicates that the selected formulation and processing parameters provided adequate interfacial stabilisation and efficient droplet disruption during homogenisation, resulting in a physically stable nanoemulsion system.

The results highlight the role of surfactant concentration in providing interfacial stabilisation, which prevents droplet coalescence during high-pressure homogenisation (Kumar et al., 2021; Singh et al., 2017). Glycerol also contributed to stability by increasing the viscosity and density of the continuous phase, slowing droplet movement and reducing creaming (Gupta et al., 2016). The chosen homogenisation pressure provided sufficient mechanical energy for droplet disruption without causing excessive thermal stress, protecting heat-sensitive bio- actives such as carotenoids and phenolics (Zhou et al., 2023).

Overall, the optimisation demonstrates that a balance among surfactant coverage, continuous-phase properties, and mechanical energy input is critical for producing a physically stable nanoemulsion with uniform droplet size. This approach also ensures cost-effectiveness by avoiding excessive surfactant or energy input while achieving desirable physicochemical characteristics, which is consistent with previous findings in plant-based nanoemulsion systems (Chong et al., 2018; Singh et al., 2017).

Accelerated Stability Studies

Nanoemulsions are thermodynamically unstable systems and therefore require stability evaluation to ensure formulation robustness (De Oca-Ávalos et al., 2017). Conventional stability testing under normal storage conditions is useful but often time-consuming. Accelerated stress testing provides an alternative approach by exposing formulations to extreme conditions, such as pH variation and elevated temperatures, to rapidly assess stability behaviour and identify potential instability mechanisms (Ali et al., 2013).

Stability of Nanoemulsion in Response to pH

pH can induce instability due to changes in the physicochemical properties of the emulsifier and the interfacial structure (Ren et al., 2021). In this study, the nanoemulsion was tested across the pH range of 2 to 6. The data obtained indicated there were no significant changes in droplet size or polydispersity index (PDI), indicating strong resistance to pH fluctuations, as shown in Table 9. These results also showed that the nanoemulsion is structurally stable, which is likely to maintain its quality during storage and use. This stability minimises the risk of emulsion separation or degradation over time, highlighting that the formulation is stable for long-term storage.

This stability is likely due to the protective interfacial layer formed by Tween® 80 and the increased viscosity from glycerol, which limits droplet movement and aggregation (Gupta et al., 2016; Kaur et al., 2024). Maintaining droplet uniformity under varying pH also helps preserve sensitive bioactive compounds like carotenoids and phenolics (Zhou et al., 2023). These results suggest that the nanoemulsion is physically and chemically stable, suitable for storage and applications in environments with varying pH.

Stability of Nanoemulsion in Response to Temperature

Temperature is a highly relevant parameter in nanoemulsion stability because it can influence droplet size, coalescence, and overall physicochemical behaviour. According to

Table 9
Effect of pH values (pH 2 to 6) on mean droplet size, polydispersity index, and cream layer of nanoemulsion

pH values	Mean Droplet Size (nm)	PDI	Presence of Creaming Layer
Control	151.8 ± 1.3^A	0.127 ± 0.021^a	Absent
2	151.2 ± 0.7^A	0.114 ± 0.022^a	Absent
3	150.0 ± 1.7^A	0.123 ± 0.001^a	Absent
4	151.7 ± 0.6^A	0.113 ± 0.018^a	Absent
5	149.5 ± 1.5^A	0.116 ± 0.016^a	Absent
6	149.5 ± 2.0^A	0.134 ± 0.004^a	Absent

Note. Different superscript letters in the same column denote statistical significance ($P < 0.05$), and the control is the untreated optimised nanoemulsion

Fu et al. (2022), elevated temperatures enhance molecular motion and the frequency of droplet collisions, which can promote coalescence and lead to larger droplet sizes. However, as shown in Table 10, the optimised nanoemulsion showed no significant changes in droplet size, PDI, or creaming when exposed to higher temperatures. This thermal stability may be attributed to the effective interfacial stabilisation provided by the surfactant and the viscosity-enhancing effect of glycerol, which reduces droplet mobility and resists coalescence. These findings indicate that the formulation is robust under thermal stress, supporting its potential for long-term storage and practical application.

Stability of Nanoemulsions in Response to Ionic Strength

Ionic strength affects nanoemulsion stability by influencing droplet interactions and emulsifier behaviour. Higher salt concentrations generally reduce electrostatic repulsion, which can promote droplet aggregation and phase separation (Li et al., 2022). In this study, the optimised nanoemulsion remained stable with no significant change in droplet size up to 1.5 mM ionic strength. Interestingly, at 2.0 mM, a slight decrease in droplet size was observed (Table 11), while the PDI remained unchanged, indicating that droplet uniformity was maintained.

This behaviour contrasts with previous reports where higher ionic strength led to droplet growth and instability (Bai & McClements, 2016; Onaizi, 2022; Qian et al., 2011). The slight reduction in droplet size observed here may be due to enhanced surfactant packing at the oil-water interface. Electrolytes can compress the electrical double layer and promote closer packing of surfactant molecules at the oil-water interface, resulting in a more compact droplet structure (Adjonu et al., 2023; Hunter et al., 2020). These results suggest that the nanoemulsion is resilient to moderate changes in ionic strength, which is beneficial for practical applications in different aqueous environments.

Table 10
Effects of heat treatment (30 to 60ºC) on mean droplet size, polydispersity index, and the cream layer of nanoemulsion

Temperature (ºC)	Mean Droplet Size (nm)	PDI	Presence of Creaming Layer
Control	151.8 ± 1.3^A	0.127 ± 0.021^a	Absent
40	151.3 ± 0.7^A	0.111 ± 0.016^a	Absent
45	151.7 ± 0.5^A	0.121 ± 0.015^a	Absent
50	151.0 ± 1.6^A	0.116 ± 0.019^a	Absent
55	151.4 ± 1.3^A	0.105 ± 0.007^a	Absent
60	152.2 ± 0.9^A	0.108 ± 0.005^a	Absent

Note. Different superscript letters in the same column denote statistical significance ($P < 0.05$), and the control is the untreated optimised nanoemulsion

Table 11
Effects of ionic strength (0.5 to 2.5mM) on mean droplet size, polydispersity index, and the cream layer of nanoemulsion

Ionic Strength (mM)	Mean Droplet Size (nm)	PDI	Presence of Creaming Layer
Control	151.8 ± 1.3^{A}	0.127 ± 0.021^{a}	Absent
0.5	150.7 ± 1.0^{A}	0.124 ± 0.014^{a}	Absent
1.0	149.4 ± 0.6^{AB}	0.114 ± 0.004^{a}	Absent
1.5	149.8 ± 1.1^{AB}	0.113 ± 0.018^{a}	Absent
2.0	146.8 ± 0.7^{B}	0.127 ± 0.012^{a}	Absent
2.5	147.5 ± 1.7^{B}	0.105 ± 0.005^{a}	Absent

Note. Different superscript letters in the same column denote statistical significance ($P < 0.05$), and the control is the untreated optimised nanoemulsion

Stability of Nanoemulsion in Response to Centrifugal Speed

Centrifugation is a widely used accelerated method to evaluate nanoemulsion stability by simulating long-term storage effects such as creaming, sedimentation, and coalescence (Preeti et al., 2023; Wu et al., 2020). Applying an increased gravitational force provides rapid insights into a formulation's structural integrity under stress.

In this study, centrifugal speeds up to 3000 rpm had no significant impact on mean droplet size. At 4000 and 5000 rpm, a slight but significant decrease in droplet size was observed (Table 12), while the PDI remained stable across all conditions, indicating consistent droplet uniformity. No creaming or phase separation was detected, highlighting the nanoemulsion's resilience. The observed size reduction may be linked to improved packing or minor reorganisation under stress.

The small droplet size (~150 nm) contributed to strong resistance against coalescence and gravitational separation, supporting the kinetic stability of the nanoemulsion (Li et al., 2025). These findings suggest that the formulation can maintain its physicochemical properties under mechanical stress, which is relevant for handling, transportation, and practical storage conditions.

Table 12
Effects of centrifugal speed (1000 to 5000 rpm) on mean droplet size, polydispersity index, and cream layer of nanoemulsion

Centrifugal Speed (rpm)	Mean Droplet Size (nm)	PDI	Presence of Creaming Layer
Control	151.8 ± 1.3^{A}	0.127 ± 0.021^{a}	Absent
1000	152.1 ± 0.9^{A}	0.135 ± 0.015^{a}	Absent
2000	150.1 ± 1.9^{AB}	0.119 ± 0.009^{a}	Absent
3000	151.9 ± 0.9^{A}	0.121 ± 0.006^{a}	Absent
4000	148.4 ± 0.6^{BC}	0.127 ± 0.020^{a}	Absent
5000	146.2 ± 0.3^{C}	0.121 ± 0.019^{a}	Absent

Note. Different superscript letters in the same column denote statistical significance ($P < 0.05$), and the control is the untreated optimised nanoemulsion

Table 13
Mean of droplet size and polydispersity index for long-term stability study

Month	0.0	2.0	3.0	4.0
Storage at 5°C				
Droplet size (nm)	$151.8 \pm 1.3^{A, a}$	$152.7 \pm 1.4^{A,a}$	$152.1 \pm 0.9^{A,a}$	$150.1 \pm 0.6^{B,a}$
PDI	$0.127 \pm 1.3^{A,a}$	$0.130 \pm 0.001^{A,a}$	$0.102 \pm 0.005^{A,a}$	$0.127 \pm 0.005^{A,a}$
Storage at 25°C				
Droplet size (nm)	$151.8 \pm 1.3^{A,a}$	$152.8 \pm 1.5^{A,a}$	$152.1 \pm 1.0^{A,a}$	$152.7 \pm 0.5^{A,a}$
PDI	$0.127 \pm 0.021^{A,a}$	$0.118 \pm 0.007^{A,a}$	$0.121 \pm 0.009^{A,a}$	$0.098 \pm 0.001^{B,a}$
Storage at 40°C				
Droplet size (nm)	$151.8 \pm 1.3^{A,a}$	$152.9 \pm 1.8^{A,a}$	$152.9 \pm 0.6^{A,a}$	$151.5 \pm 1.3^{AB,a}$
PDI	$0.127 \pm 0.021^{A,a}$	$0.134 \pm 0.012^{A,a}$	$0.107 \pm 0.005^{A,a}$	$0.125 \pm 0.013^{A,a}$

Note. Different superscript letters in the same column denote statistical significance ($P < 0.05$)

Long-term Stability Study

Table 13 shows that the storage temperature and duration affect the mean droplet size of the nanoemulsion. The nanoemulsion had a consistent droplet size at the start (zeroth month). After three months, the droplet size remained stable across all temperatures. However, the droplet size at 25°C was noticeably smaller by the fourth month than at the other temperatures. The table also shows the PDI over the four months at 5°C, 25°C, and 40°C. No significant changes in PDI were seen during the first three months at any temperature. However, after four months, the PDI at 25°C was significantly lower than at the other temperatures. These results and the mean droplet size data suggest that the nanoemulsion remained physically stable for four months, confirming the stability of the RSM-optimised formulation, as there were no signs of instability, such as Ostwald ripening, coalescence, flocculation, or creaming (Kampa et al., 2022).

CONCLUSION

In this study, a red palm oil-based oil-in-water nanoemulsion containing *Strobilanthes crispus* extract was successfully optimised using response surface methodology. The optimum formulation consisted of 5.06% (w/w) Tween® 80, 10.4% (w/w) glycerol, and a homogenisation pressure of 617.1 bar, producing a nanoemulsion with a droplet size of 151.8 ± 1.3 nm, PDI of 0.127 ± 0.021, and no observable creaming.

Accelerated and four-month storage stability studies confirmed consistent physicochemical stability across varying pH, ionic strength, temperature, and centrifugation conditions. These findings indicate that the developed nanoemulsion is a stable delivery system for lipophilic bioactive compounds.

Further studies such as biological activity evaluation, skin penetration, and advanced characterisation (e.g., zeta potential and interfacial tension) can be explored in future work.

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