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Optimization of Nanoemulsion Formulation from the Aqueous Fraction of Dayak Onion (Eleutherine bulbosa (Mill.) Urb) Tuber Extract Using Variations of Olive Oil, Tween 20, and PEG 400

Husnul Warnida*, Umi Erabiatul Azira, Supomo, Rizqa Akhriana Putri, Eka Siswanto Syamsul

Pharmacy Program, Sekolah Tinggi Ilmu Kesehatan Samarinda, Kalimantan Timur, Indonesia

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*Corresponding author.
E-mail: *husnulwarnida@gmail.com*

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ABSTRACT

Dayak onion (*Eleutherine bulbosa* (Mill.) Urb) is known for its antioxidant, anti-inflammatory, and antimicrobial activities, attributed to its flavonoid, alkaloid, and saponin contents. However, the bioavailability of these active compounds is limited due to poor stability and absorption. To enhance their effectiveness, nanoemulsion formulations were developed. This study aimed to investigate the effects of varying concentrations of olive oil, Tween 20, and PEG 400 on the physical characteristics of nanoemulsions containing the water fraction of Dayak onion bulb extract, and the optimization was performed using a D optimal mixture design (Design Expert 13) with three independent variables: olive oil (1–11%), Tween 20 (1–11%), and PEG 400 (1–16%). The extract concentration was kept constant at 1%. The responses evaluated were pH, creaming index, transmittance, droplet size, polydispersity index, and zeta potential. The optimum formula was selected based on the highest desirability value. The optimal formulation exhibited a stable pH (5.00–6.12), a low creaming index (0–0.3), an average particle size of 308 nm with a polydispersity index of 0.51, and a zeta potential of –38.6 mV, indicating good electrostatic stability. However, the transmittance percentage did not reach the ideal standard (>80%). Significant differences were observed between predicted and experimental values for the creaming index and transmittance, and changes in the creaming index occurred during 30 days of storage. Further optimization is recommended to enhance the stability and clarity of the nanoemulsion.

Keywords: Dayak onion; *Eleutherine bulbosa*; Emulsified; Bioavailable; Water Fraction

INTRODUCTION

Medicinal plants have long been utilized as natural remedies for various diseases. Dayak onion (*Eleutherine bulbosa* (Mill.) Urb) is one such plant known for its rich content of bioactive compounds, including flavonoids, alkaloids, and saponins, which exhibit antioxidant, anti-inflammatory, and antimicrobial properties.[1] Despite its promising therapeutic potential, the bioavailability of flavonoids from Dayak onion bulbs is limited due to poor solubility and extensive metabolism in the liver and intestines.[1]

Nanoemulsion-based formulations, particularly oil-in-water (o/w) systems, have been developed to enhance the

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solubility, stability, and absorption of bioactive compounds.[2] Their small droplet size (<1000 nm) allows easier penetration through biological membranes, improving drug absorption and accelerating gastrointestinal uptake.[3,4] One effective method for nanoemulsion preparation is the Self-Nanoemulsifying Drug Delivery System (SNEDDS), which consists of oil, surfactant, and cosurfactant components.[5]

In nanoemulsion optimization, the aqueous fraction plays a key role in extracting polar compounds such as flavonoids and phenolics, while the oil phase acts as a lipophilic carrier ([6,7]). Olive oil is often employed to improve formulation stability and bioavailability. Tween 20, with a high Hydrophilic-Lipophilic Balance (HLB) value of 16.7, serves as a surfactant that enhances solubility. To further reduce interfacial tension, PEG 400 is used as a cosurfactant, improving dispersion and system stability.[6,7]

Determining the optimal nanoemulsion formula requires evaluating parameters such as particle size, stability, and bioavailability. The Design Expert software can be utilized to analyze the interactions among formulation components and to identify the most effective composition for achieving stable and efficient nanoemulsions.[8]

Therefore, this study aims to determine the effect of varying concentrations of olive oil, Tween 20, and PEG 400 on the physical characteristics of the *Dayak onion* bulb extract nanoemulsion and to obtain the optimal formulation using the Design Expert 13 method. The optimized formulation is expected to enhance the solubility, stability, and bioavailability of the bioactive compounds in Dayak onion extract for potential therapeutic applications.

METHODS

Equipment and materials

The equipment to be used in this study consists of measuring cups (Pyrex® and Iwaki®), digital scales (OHAUS®), maserators (IKA®), rotary evaporators (IKA®), viscometers (Brookfield LVT®), micropipettes (Thermo®), pH meter, homogenizer (Ultra Turrax®), stirring rod, porcelain dish, aluminum foil, glass jar, spatula, watch glass, measuring flask, filter paper and falcon tube, Size Analyzer (PSA), hot plate, magnetic stirrer, vortex, glass jars, 40 mesh sieve, Erlenmeyer flasks, UV-Vis spectrophotometer, blender, Buchner funnel, vacuum, and separating funnel.

The materials used in this study were Dayak onion bulb extract, olive oil, Tween 20, PEG 400, 70% ethanol, and distilled water.

Preparation of *Eleutherine bulbosa* (dayak onion) simplicial

Identification was carried out at the Laboratory of Ecology and Tropical Forest Biodiversity Conservation, Faculty of Forestry, Mulawarman University, Samarinda.

Preparation of *Dayak Onion* Bulb Simplicia

The part of *Dayak onion* (*Eleutherine bulbosa* (Mill.) Urb) used in this study was the bulb, with a total sample weight of approximately 16.5 kg. The collected bulbs were subjected to wet sorting by separating them from the roots and leaves, followed by washing under running water and draining. The bulbs were then sliced and dried. Once completely dry, the simplicia was ground using a blender and sieved through a 40-mesh sieve. The powdered simplicia was stored in a clean, tightly sealed glass container.[1]

Extraction Using the Maceration Method

A total of 250 grams of Dayak onion simplicia powder was macerated with 70% ethanol solvent in a ratio of 1:10 (b/v), using 2.5 liters of solvent. The mixture was stirred with a macerator for 6 hours and left to stand for 18 hours to obtain the first filtrate (macerate). The remaining residue was re-macerated twice with 70% ethanol in a ratio of 1:5 (b/v), using 1.25 liters of solvent for each repetition. All filtrates were combined and concentrated using a rotary evaporator, followed by

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evaporation in a water bath to obtain a thick Dayak onion bulb extract.

The extract yield was calculated as the percentage ratio (w/w) between the weight of the obtained extract and the initial weight of the simplicia powder used.[10] The yield percentage was calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of simplicia powder (g)}}{\text{Weight of extract (g)}} 100\,\%$$

Fractionation

A total of 10 grams of thick extract was dissolved in 10 mL of distilled water and subjected to separation using a separatory funnel with 100 mL of n-hexane or chloroform. The mixture was repeatedly shaken until homogeneous, with a ratio of liquid extract to solvent of 1:1.[9] The mixture was then allowed to stand until two distinct layers formed: the lower layer consisted of water, while the upper layer was n-hexane or chloroform. The fractionation process was repeated twice. The obtained liquid fractions were evaporated using a water bath to yield a concentrated fraction. The percentage yield of the fraction was then calculated.

Formulation of Nanoemulsion Preparation from Water Fraction of Dayak Onion Bulbs

D-optimal mixture design was implemented using Design Expert 13 (Stat-Ease, Inc.). Three independent variables were selected: olive oil (A), Tween 20 (B), and PEG 400 (C). Their concentration ranges were set based on preliminary trials: A = 1–11%, B = 1–11%, and C = 1–16% (w/w of total formulation). The extract concentration was fixed at 1% w/w. The design generated 10 experimental runs (Table 1).

Table 1. Nanoemulsion preparation formula obtained from Design Expert for 100 ml preparation.

Table 1. Formula of Dayak onion nanoemulsion

Formula	Extract (%)	*Olive oil (%)*	Tween 20(%)	PEG 400(%)
1	1.00	6.00	6.00	1.00
2	1.00	7.67	2.67	3.50
3	1.00	11.00	1.00	1.00
4	1.00	1.00	11.00	1.00
5	1.00	4.33	4.33	6.00
6	1.00	2.67	7.67	3.50
7	1.00	6.00	1.00	8.50
8	1.00	1.00	1.00	16.00
9	1.00	1.00	6.00	8.50
10	1.00	2.67	2.67	11.00

One gram of the aqueous fraction was dissolved in 10 mL of 70% ethanol in a 10 mL volumetric flask up to the mark. Then, 1 mL of the aqueous fraction was mixed with olive oil according to the formulation and stirred using a magnetic stirrer at 800 rpm for 15 minutes. Tween 20 was added and stirred again at 800 rpm for 15 minutes. Subsequently, PEG 400 was incorporated and the mixture was stirred at 800 rpm. Finally, distilled water was added to a total volume of 100 mL, followed by particle size reduction using an ultrasonic device for 30 minutes until a nanoemulsion or a clear preparation was obtained.[19]

The optimization of the nanoemulsion formulation was guided by predefined criteria for each response parameter. The target for pH was set within the range of 4.5–7.0 to ensure physiological

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compatibility and stability. The creaming index was to be minimized, with an acceptable upper limit of 20%, as lower values indicate better physical stability. Transmittance was maximized to achieve optical clarity, ideally approaching 100%, which reflects small droplet sizes and a homogeneous system. Droplet size was minimized, with a desired range of 50–500 nm, to confirm the formation of a true nanoemulsion. The polydispersity index (PDI) was also minimized to ensure uniform droplet distribution, with values below 0.5 considered acceptable. Lastly, the absolute zeta potential was maximized (target <-30 or >30 mV) to promote electrostatic repulsion and prevent coalescence. Importance values were assigned according to each parameter's impact on formulation quality, with creaming index and droplet size receiving the highest priority.

Determination of the Optimal Nanoemulsion Formula of Dayak Onion Bulb Aqueous Fraction

Numerical optimization was performed using the desirability function with the following criteria: minimize droplet size and PDI, maximize transmittance and zeta potential, and achieve creaming index = 0 and pH within 4.5–7.0. The formulation with the highest desirability was selected as the optimum. The optimal formula was verified by preparing the nanoemulsion again and testing the same response parameters. Verification data were compared with the predicted values from the software and analyzed using an Independent Sample T-Test to assess significant differences.

Evaluation of Nanoemulsion Characteristics and Stability pH Measurement

The electrode was calibrated using standard buffer solutions at pH 4.0 (acid), 6.8 (neutral), and 9.3 (base), rinsed with distilled water, and dried with tissue paper. Calibration was repeated for all three standards. The electrode was then immersed in the sample until a stable pH reading appeared. Measurements were performed in triplicate.[11]

Creaming Index Test

Nanoemulsion samples (10 mL) were placed in Falcon tubes, capped, and stored upright or horizontally. Stability was evaluated by measuring the creaming index to determine phase separation. Unstable samples typically separate into two layers.[12]

Transmittance (%) Test

One milliliter of the nanoemulsion was diluted to 100 mL with distilled water. The transmittance was measured at 650 nm using a spectrophotometer with distilled water as a blank. Transmittance values close to 100% indicate good clarity, suggesting that the nanoemulsion droplets are within the nanometer range, as visually observed by the transparency of the system.[12,17]

Droplet Size and Polydispersity Index (PDI) Measurement

Droplet size was measured using a Particle Size Analyzer (PSA) with dynamic light scattering (DLS). Ten milliliters of the sample were placed in a clean cuvette to avoid interference. The sample was then analyzed, with nanoemulsions considered successfully formed if the droplet size was <200 nm and the PDI <0.5, indicating stability against particle collision and gravitational separation.[18,19]

Zeta Potential Measurement

Zeta potential was measured using a Zetasizer at 25°C. Five milliliters of nanoemulsion were placed in a cuvette, and the measurement was conducted within 15 minutes. A nanoemulsion formula is considered stable if it has a high zeta potential, typically above ±30 mV.[13,14]

Data Analysis

Statistical analysis of the quantitative data (pH, viscosity, creaming index, transmittance, droplet size, zeta potential, and polydispersity index) was conducted with Design Expert 13.

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Subsequently, an Independent Sample T-Test was utilized to determine if significant differences existed in the pH, transmittance, and creaming index values between the experimental formulation and the formula predicted as optimal by Design Expert.[15,16]

RESULTS AND DISCUSSION

The simplicia powder, sieved through a 40-mesh, yielded 3 kg and was divided into 12 jars, each containing 250 g. The powder was extracted by maceration using 70% ethanol (2.5 L for the first extraction and 1.25 L for re-maceration). The simplicia was soaked in glass jars for 24 hours, stirred for the first 6 hours, and left undisturbed for 18 hours. The extract was vacuum-filtered, and the residue was re-macerated once. Filtrates were concentrated using a rotary evaporator at 50°C and further dried in a water bath, yielding 460.46 g of thick extract (15.34%).

Fractionation was performed to separate compounds based on polarity using n-hexane (non-polar) and chloroform (semi-polar). The aqueous fraction was concentrated in a water bath, and its yield was calculated.[20] The percentage yields of the Dayak onion bulb fractions are presented in Table 2.

The weighing and calculation results showed three concentrated fractions: the n-hexane fraction weighed 8.68 g with a yield of 34.72%, the chloroform fraction weighed 16.13 g with a yield of 64.52%, and the aqueous fraction weighed 18.34 g with a yield of 73.36%. These results indicate that the majority of compounds in the ethanol extract of Dayak onion bulbs are polar.

The nanoemulsion consists of oil, water, surfactant, and co-surfactant phases. In this study, the developed nanoemulsion is an oil-in-water type, with olive oil serving as the internal phase and water as the external phase. The co-surfactant, PEG 400, aids Tween 20 in reducing surface tension, thereby enhancing the stability of the nanoemulsion.

The pH values of the 10 nanoemulsion formulas ranged from 5.00 to 5.76, which is classified as weakly acidic. These values fall within the ideal range for nanoemulsion formulations (4.5–7.0)[12]. The lowest pH (5.00) was observed in Formulas 6, 8, and 9, while the highest pH (5.76) was observed in Formula 1. This variation is likely due to differences in composition, particularly the ratio of olive oil to Tween 20, which is neutral. Formulas with higher Tween 20 content tend to have pH closer to neutral. Formula 8, with a pH of 5.00, exhibited the best stability, with a 0% creaming index and high percent transmittance (73.55%). This suggests that a pH around 5.00 is optimal for this nanoemulsion system, where the oil-water interfacial stability is maximized. The combination of Tween 20 and PEG 400 forms a stable interfacial layer, helping maintain the system's pH. Achieving this pH range is critical for ensuring stability during storage. Factors such as the use of olive oil with low free fatty acid content (<0.8% for extra virgin) contribute to acidity, while Tween 20, with a neutral pH (6–8), stabilizes the overall system. PEG 400 concentration showed no significant effect on pH changes, as indicated by the relatively stable pH range.

The transmittance test measures the clarity of the nanoemulsion, indicating the degree of dispersion. Percent transmittance was measured using a UV-Vis spectrophotometer at 650 nm.[12]

Table 2. Yield Calculation Results

Sample	Initial weight (g)	Final weight (g)	Rendemen (%)
Ethanol extract	3000	460.4	15.34
Water fraction	25.00	18.34	73.36
Chloroform fraction	25.00	16.13	64.52
N-hexane fraction	25.00	8.68	34.72

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Table 3. Results of Nanoemulsion Preparation Evaluation

Formula	pH	Creaming Index (%)	% Transmitant
1	5.76	0	0.03
2	5.38	20.00	40.93
3	5.53	20.00	51.65
4	5.45	1.00	0.50
5	5.38	5.00	16.57
6	5.02	5.00	38.60
7	5.30	10.00	54.81
8	5.00	0	73.55
9	5.00	0	68.98
10	5.20	0	29.23

As shown in Table 3, all formulas produced dispersions, though some appeared visually turbid with transmittance below 80%, indicating that none fully met the desired criteria. High transmittance values (>80%) indicate very small droplet sizes, allowing light to pass through easily.[13] Formulas with high transmittance (>50%), such as Formula 8 (73.55%) and Formula 9 (68.98%), benefited from PEG 400 acting as both a solvent and stabilizer, combined with lower olive oil content, which minimized disruption. Adjusting the ratio of olive oil and PEG 400 resulted in a stable system with high transmittance. Some formulas, such as Formula 1 (0.03%) and Formula 4 (0.50%), had low transmittance due to system instability, phase separation, and stirring methods.

The creaming index (CI) evaluates emulsion stability by measuring phase separation between the dispersed and continuous phases. CI is calculated as the percentage of the clear layer relative to the cream layer. Emulsion stability is inversely proportional to CI; lower CI indicates higher stability. Table 3 shows CI values for the 10 nanoemulsion formulas, ranging from 0% to 20%. Formula 1, with balanced olive oil (6%) and Tween 20 (6%) and low PEG 400 (1%), demonstrates the importance of matching surfactant HLB to the oil phase for optimal stability.[15] Formulas 8, 9, and 10 exhibited the best stability with CI = 0%, consistent with previous studies highlighting PEG 400's stabilizing role via increased continuous phase viscosity and steric layer formation at the oil-water interface.[14] Formulas 3 and 4 showed the highest CI (20%), likely due to high olive oil content (Formula 3 = 7.67 mL, Formula 4 = 11.60 mL), which increases the density difference between oil and water phases, accelerating creaming.[15] Formulas with low oil content (<2.67 mL), such as Formulas 1 and 5 (1.00 mL each), achieved optimal stability (CI = 0%). Formulas with Tween 20 ≥10 mL (Formulas 8–10) also achieved CI = 0%, as Tween 20 reduces interfacial tension, forms a steric protective layer, and enhances electrostatic repulsion between droplets. CI measurements were conducted in 10 mL Falcon tubes.

The optimal nanoemulsion of the Dayak onion bulb aqueous fraction was determined using Design Expert software based on the evaluation of the 10 initial formulas. The software recommended the best formula along with its characteristics and stability evaluation, as shown in Figure 1.

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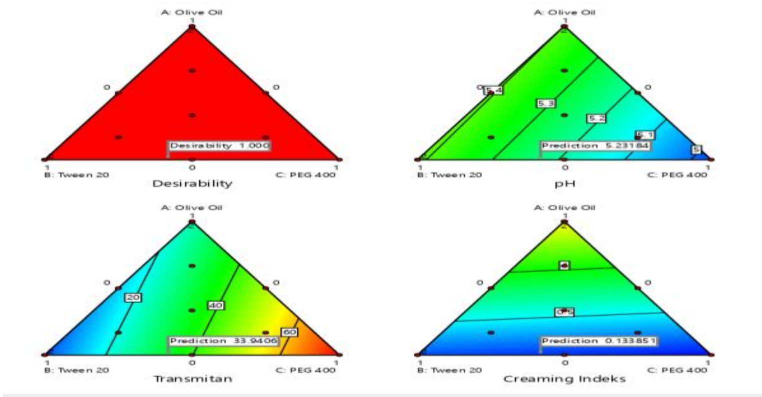

Figure 1. Design Expert Prediction Formula

Table 5. Optimum Nanoemulsion Formula

Recommendation	Olive Oil (%)	Tween 20 (%)	PEG 400 (%)
1	1.21	6.70	7.12

The physical properties of the Dayak onion bulb water fraction nanoemulsion were analyzed using Design Expert 13, yielding a desirability value of 1. A desirability value close to 1 indicates that the program can produce a formulation approaching the target parameter range, with higher values reflecting better product quality. The optimal formula recommended by Design Expert was prepared as a nanoemulsion in three replicates. Experimental results were compared with the software predictions using an independent sample t-test to determine significant differences between the experimental and predicted means. The characteristics of the optimal formula and its stability (pH, % transmittance, and creaming index) on day 1 and day 30 are presented in the following table.

Table 6. Results of the Optimum Formula for Water Fraction Nanoemulsion

Response	Replication 1	Replication 2	Replication 3	Mean
pH	5.26	5.33	6.12	5.57±0.47
Creaming index	0	0	0	0
Transmitant (%)	78.63	53.89	63.63	65.38±12.46
Droplet size (nm)	304.1	308.2	311.5	307.93±3.71
Polydispersity index	0.59	0.43	0.50	0.51±0.08
Zeta Potensial (mV)	-37.4	-38.5	-39.9	-38.6±1.25

The pH of the optimal nanoemulsion formula, replicated three times, ranged from 5.26 to 6.12, which is safe for oral preparations and within the acceptable pH range. The creaming index (CI) was 0% in all replicates, indicating

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excellent emulsion stability and no phase separation, demonstrating the effectiveness of the surfactant and co-surfactant in reducing oil–water interfacial tension.

Percent transmittance was measured using a UV–Vis spectrophotometer at 650 nm. Values varied across replicates (78.63%, 53.89%, and 63.63%) due to differences in magnetic stirrer equipment and processing time. These results indicate that the nanoemulsion was not fully transparent, as an ideal range is 90–100% for optical clarity.

Particle size analysis showed average droplet sizes of 307.93 nm, meeting the nanoemulsion criterion of <1000 nm. However, the polydispersity index (PDI) averaged 0.514, exceeding the ideal <0.5, indicating a moderately heterogeneous particle distribution with potential for aggregation.

Zeta potential measurements revealed high negative values (−37.4, −38.5, −39.9 mV; average −38.6 mV), confirming strong electrostatic repulsion and good colloidal stability, which prevents particle aggregation and maintains system stability.

Table 7. Results of the Optimum Formula Stability Test

Response	Day-1			Mean	Day-30			Mean
	R1	R2	R3		R1	R2	R3	
pH	5.26	5.33	6.12	5.57±0.47	4.94	4.32	5.71	4.99± 0.69
% Transmittant	78.63	53.89	63.63	65.40±12.46	69.40	73.55	68.92	70.6±2.54
Creaming index (%)	0	0	0	0	3.00	1.00	1.00	1.66± 0.94

Stability of the optimal air fraction nanoemulsion was evaluated over 30 days. After storage, pH ranged from 4.94 to 5.71, indicating a slight decrease compared to day 1, but still within acceptable limits. Creaming index (CI) after 30 days ranged from 0 to 3.00 (average 0.166± 0.94).

Percent transmittance (%) showed variability among replicates on day 1: 78.63% (R1), 53.89% (R2), and 63.63% (R3). Over 30 days, R1 decreased to 73.55% (indicative of slight instability or droplet aggregation), while R2 increased to 68.92%, possibly due to spontaneous homogenization or redistribution of particles during storage.

Normality of the data was tested using the Shapiro–Wilk test (appropriate for <50 samples), yielding $p > 0.05$, indicating the data were normally distributed. Results for day 1 and day 30 in Table 8.

Table 8 shows that the independent t-test between Design Expert predictions and experimental data revealed significant differences in two out of three parameters. %Transmittance ($p = 0.012$) and Creaming Index ($p = 0.025$) differed significantly, while pH ($p = 0.224$) showed no significant difference, likely due to its deterministic nature and dependence on buffer capacity.

Stability analysis (Table 11) indicated that only the Creaming Index changed significantly ($p = 0.034$) after 30 days, whereas pH ($p = 0.300$) and %Transmittance ($p = 0.515$) remained stable. Minor pH variations (0.5–0.6 units) suggest an effective buffer system, and unchanged %Transmittance reflects stable particle size and optimal emulsifier function. Creaming observed in the Creaming Index indicates phase separation, highlighting the need for further formulation optimization.

Table 8. Summary of statistical comparisons for the optimum formulation and stability

Parameter	Comparison	Test used	p-value	Conclusion
pH	Predicted vs. Experimental	Independent t-test	0.224	Not significantly different
%Transmittance	Predicted vs. Experimental	Independent t-test	0.012	Significantly different
Creaming index	Predicted vs. Experimental	Mann–Whitney	0.025	Significantly different
pH	Day 1 vs. Day 30	Independent t-test	0.300	Not significantly different
%Transmittance	Day 1 vs. Day 30	Independent t-test	0.515	Not significantly different
Creaming index	Day 1 vs. Day 30	Mann–Whitney	0.034	Significantly different

The Mann–Whitney test was used for creaming index because the data were not normally distributed (Shapiro–Wilk $p < 0.05$). The significant increase in creaming index after 30 days indicates gradual phase separation, suggesting that the current surfactant system may require further optimization for long-term stability.

Statistical analysis using the Mann–Whitney test was conducted to evaluate the effectiveness of the optimum formulation and stabilizer system in preventing creaming. The optimum formulation showed a significance value of 0.025 ($p < 0.05$), indicating that phase separation was not fully prevented during storage. The stabilizer system yielded a significance value of 0.034 ($p < 0.05$), suggesting that while statistically significant, its stability was generally adequate but not yet fully optimal.

CONCLUSION

The variation in the concentrations of olive oil, Tween 20, and PEG 400 affected the physical characteristics of the nanoemulsion, particularly pH (5.00–5.76), % transmittance (0.03–73.55%), and creaming index (0–20%). The optimal formulation (1.21% olive oil, 6.70% Tween 20, 7.12% PEG 400) exhibited good physical stability, with a creaming index of 0%, particle size of 307.93 nm, zeta potential of -38.6 mV, and a PDI of 0.51. However, the % transmittance of 65.38% remained below the ideal criterion (>80%), indicating that further optimization is required.

Conflict of Interest
The authors declare no conflict of interest.

Authors' Declaration
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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