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Optimization of Xanthorrhizol Nanoemulsion Formulation Using The Design of Experiment Approach (Box-Behnken Methods)

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ABSTRACT: Nanoemulsion is an effective drug delivery system to enhance the penetration of active substances. Xanthorrhizol (XNT) shows potential as an anti-aging agent with collagenesis activity and photo-aging inhibition, but its lipophilic nature limits skin penetration and reduces its effectiveness in reaching the stratum corneum. Therefore, this study aims to optimize the formulation of xanthorrhizol nanoemulsion using the Design of Experiment (DoE) statistical approach with the Box-Behnken Design (BBD) method on the Design Expert® software. The optimization in this study involved three independent variables (X): (X_1) the concentration of Smix (Tween 80 and PEG 400), (X_2) stirring time, and (X_3) stirring speed. The responses (Y) evaluated to determine the optimization outcome were (Y_1) percent transmittance (%T) and (Y_2) pH. Based on the optimization results, the base formula with the amount of Smix 60%, stirring speed 875 rpm and stirring time 15 minutes gave a 98.4%T response and pH 7.05. The xanthorrhizol nanoemulsion formula was yellow in color and had a distinctive xanthorrhizol odor. The formulation exhibited a globule size of 13.76 ± 0.2 nm, a polydispersity index of 0.059 ± 0.000, and a zeta potential of −48.03 ± 1.423 mV. It also showed 95.5 ± 0.31% transmittance, had a pH of 7.12 ± 0.01, and was classified as an oil-in-water (O/W) nanoemulsion. The nanoemulsion remained stable after freeze-thaw and centrifugation tests, with an entrapment efficiency of 58.6%. Based on these findings, the developed formulation can be considered a promising nanoemulsion system for xanthorrhizol delivery.

Keywords: **nanoemulsion; xanthorrhizol; design of experiment; box benhken.**

Introduction

Skin is a major organ with a large surface area, making it a potential pathway in drug delivery systems. However, the skin also serves as an effective barrier in limiting the penetration of therapeutic compounds, especially those with large molecular sizes or unfavorable physicochemical properties. This can affect the effectiveness of drug delivery to the target site [1].

One of the technological approaches developed to overcome these obstacles is nanotechnology. These systems are able to deliver active compounds at minimal concentrations and target specific sites of action [2]. One effective nanotechnology system is nanoemulsion, which is a transparent (translucent) emulsion system with a globule size below 200 nm [3]. Nanoemulsions are able to increase the solubility and local concentration of active compounds in lipophilic components [4], so they are often utilized in topical drug delivery systems, including anti-aging product [5].

Anti-aging product aim to reduce signs of aging such as wrinkles and fine lines and increase the moisture level of the skin [6]. Anti-aging activity is influenced by factors such as the enzymes Matrix Metalloproteinase-1 (MMP-1) and procollagen-1, which play a role in maintaining skin elasticity and structure [7]. One compound with anti-aging potential is xanthorrhizol, which comes from the rhizome of temulawak (*Curcuma xanthorrhiza* Roxb.) and has been shown to reduce MMP-1 and increase procollagen-1, even more effectively than epigallocatechin-3-O-gallate (EGCG) [8].

Nanoformulation approaches have been investigated to enhance the solubility, stability, and delivery efficiency of extract from *Curcuma xanthorrhiza* [9]. Previous studies have reported that SNEDDS-based systems were capable of producing particle sizes below 100 nm and demonstrated acceptable physical stability without phase separation, indicating their potential as suitable nanocarrier platforms [10]. Additionally, the application of the Box–Behnken design in formulation development yielded nano-sized systems with favorable dispersion characteristics. However, despite these promising outcomes, existing research

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remains limited, as critical parameters such as entrapment efficiency, long-term stability, release behavior, and biological activity were not evaluated. The absence of these assessments restricts the comprehensive understanding of the formulation's performance and therapeutic potential. Therefore, further research is required to develop and characterize a nanoemulsion system that not only meets physicochemical criteria but also demonstrates functional and biological relevance to support its application in pharmaceutical development.

This study used xanthorrhizol as an active ingredient in nanoemulsion preparations, with a Design of Experiments approach. This approach is an optimization technique that allows testing several variables simultaneously, making it more efficient in time, cost, and providing a better understanding of the interactions between variables [11]. This study aims at optimizing the amount of Smix, stirring time and speed in xanthorrhizol nanoemulsion formulation using Behnken Box Design (BBD) method. This study utilized xanthorrhizol as the active ingredient in a nanoemulsion formulation and employed a Design of Experiments (DoE) approach using the Box–Behnken Design (BBD). BBD was selected because it enabled simultaneous evaluation of multiple formulation variables while requiring fewer experimental runs, making the process more time, cost-, and material-efficient. Additionally, the method provided valuable insight into both individual factor effects and variable interactions, which is essential for optimizing nanoemulsion systems.

Methods

Materials

The materials used include xanthorrhizol (Javaplant, Indonesia), Tween 80 (Kisbiokim Medilab, Indonesia), PEG 400 (Sigma Aldrich, USA), grapeseed oil (Kimiapedia, Indonesia), methylene blue (Bratachem, Indonesia), ethanol (Bratachem, Indonesia), and distilled water.

Procedure

Optimization of Nanoemulsion Formula Base

Nanoemulsion consists of grape seed oil (GSO), Smix (Tween 80 and PEG 400), and distilled water. Optimization prepared with mechanical stirring, where Tween 80 and PEG 400 were stirred for 5 minutes, then the oil phase was added slowly and continued stirring for 10 minutes. Distilled water was dripped slowly while stirring, then stirring continued according to the time recommended by Design Expert in Table 1. The nanoemulsion formula was then tested for percent transmittance and pH to determine the optimal formula.

The data obtained were entered into the response analysis table in Design Expert Version 23.1 software. with the BBD method. The factors considered were the amount of Smix (X_1), stirring time (X_2), and stirring speed (X_3) with analytical responses in the form of

Table 1. Experimental design for box benhken method and experimental responses.

Formula	Oil (%)	Water (%)	Factor			Response	
			X1	X2	X3	Y1	Y2
F1	10	37.5	52.5	1500	15	0.375	6.81
F2	10	45	45	250	37.5	0.162	6.43
F3	10	37.5	52.5	875	37.5	0.302	6.93
F4	10	30	52.5	875	37.5	0.296	6.94
F5	10	30	60	875	15	98.936	7
F6	10	37.5	52.5	250	60	0.311	7.08
F7	10	37.5	45	1500	37.5	0.397	6.64
F8	10	30	60	250	37.5	97.916	7.18
F9	10	45	45	875	15	0.215	6.23
F10	10	37.5	60	875	60	96.733	7.17
F11	10	37.5	52.5	250	15	0.171	6.69
F12	10	37.5	52.5	875	37.5	0.705	7.06
F13	10	30	45	875	60	0.165	6.97
F14	10	45	60	1500	37.5	99.286	7.08
F15	10	45	52.5	1500	60	0.51	6.99

Description: X1 = Smix (%); X2 = Stirring speed (RPM); X3 = Stirring time (minutes); Y1 = transmittance (%); and Y2 = pH.

Table 2. Coded levels and actual values of independent variables used in the optimization.

Symbol	Independent variable	Actual level at coded factor levels		
		-1	0	+1
X_1	Smix concentration	45	52.5	60
X_2	Stirring time	15	37.5	60
X_3	Stirring speed	250	875	1500

percent transmittance (Y_1) and pH (Y_2). The detailed parameter levels used in this study are presented in Table 2. The expected response criteria are in accordance with the set ranges of 90-99.9% for percent transmittance and 4.5-8 for pH, respectively. The nanoemulsion base formula selected as the optimum formula is the one with the highest desirability index. The experimental data were analyzed using Design-Expert Version 23.1 software with the Box–Behnken Design (BBD) approach. Three formulation factors were evaluated: Smix concentration (X_1), stirring time (X_2), and stirring speed (X_3), while the measured responses consisted of percent transmittance (Y_1) and pH (Y_2). The target ranges for optimization were set at 90–99.9% for percent transmittance and 4.5–8 for pH. The optimum nanoemulsion formula was determined based on the highest desirability index.

Preparation of Xanthorrhizol Nanoemulsion

The optimal nanoemulsion formula was prepared by mechanical stirring using a magnetic stirrer. Xanthorrhizol at a concentration of 0.1% was first incorporated into the oil phase by mixing it with grape seed oil (GSO). The mixture of Tween 80 and PEG 400 was stirred for 5 minutes at 875 rpm, then the oil phase was added and stirred for 10 minutes. After the mixture was homogeneous, distilled water was slowly dripped and stirring was continued for 15 minutes.

Evaluation of Xanthorrhizol Nanoemulsion

Organoleptic Test

Evaluation has been performed by visually observing the color, odor, and consistency [12].

Globule Size, Polydispersity Index (PDI), and Zeta Potential Tests

Measurement of globule size, polydispersity index (PDI), and zeta potential was performed using a Particle Size Analyzer (Horiba Scientific, Nanoparticle Analyzer SZ-100, Japan), which operates based on dynamic light scattering (DLS) to analyze droplet characteristics. For analysis, 1 mL of the nanoemulsion sample was diluted in 250 mL of distilled water and placed into a measurement cuvette prior to testing [13].

pH test

The pH measurement was performed by calibrating the pH meter first, then the pH meter (Mettler Toledo, USA) electrode was dipped into the xanthorrhizol nanoemulsion.

Percent Transmittance Test

The evaluation was performed using UV-Vis spectrophotometry (Shimadzu UV-1700, Japan), where distilled water was used as a blank and measured at a wavelength of 650 nm [14].

Nanoemulsion Type Test

Nanoemulsion type testing uses dilution and staining methods. In the dilution method, the nanoemulsion that has been made is diluted with distilled water. Meanwhile, the coloring method was carried out by dripping methylene blue on formula [15].

Stability Test

Stability test was conducted using 2 methods, which were centrifugation test and freeze and thaw cycle. The centrifugation test was carried out at 3500 rpm for 30 minutes, while the freeze and thaw test involved storing the preparation at -5°C and 25°C for 24 hours over three cycles. Observations have included organoleptic (shape, phase separation, and coalescence) and quantitative (percent transmittance and pH) [16].

Validation of the Analytical Method

The UV–Vis spectrophotometric method used to quantify xanthorrhizol in the nanoemulsion formulation was validated according to International Conference on Harmonization (ICH) guidelines. Before method validation was performed, the maximum absorption wavelength was first determined. Xanthorrhizol was dissolved in 96% ethanol and analyzed using a UV–Vis spectrophotometer (Shimadzu UV-1700, Japan).

Entrapment Efficiency

The entrapment efficiency was determined using the centrifugation method. A total of 2 mg of the nanoemulsion was transferred into a 10 mL centrifuge tube and diluted with 4 mL of distilled water (sample-to-solvent ratio 1:2), then centrifuged at 3500 rpm for 30 minutes. After centrifugation, 3 mL of the obtained filtrate was collected and quantified as free (non-encapsulated) xanthorrhizol.

The remaining residue and filtrate were then diluted with 9 mL of ethanol and analyzed using a UV–Vis spectrophotometer (Shimadzu UV-1700, Japan) at the maximum absorbance wavelength of xanthorrhizol. The entrapment efficiency (EE%) was calculated using the following equation:

$$\textbf{Entrapment efficiency} = (Qt - Qs)/Qt \times 100\ \%$$

Desc:
Qt: Absorbed xanthorrhizol content
Qs: Free xanthorrhizol content .

Result and Discussion

Nanoemulsion Base Formula Optimization Results

The variation of Smix amount in nanoemulsion base formula optimization ranged from 45-60%, stirring time 15-60 minutes, and stirring speed 250-1500 rpm. From the combination of these factors, 15 formula variations were obtained as shown in Table 1. The results showed that formula F14 had the highest percent transmittance (99.286%), while formula F2 had the lowest transmittance (0.162%). Formula F9 has the lowest pH (6.23) and formula F10 the highest pH (7.17).

The significant factor screening data were analyzed at a significant degree of 95% ($\alpha = 0.05$). Based on the obtained p-values, the percent transmittance response (Y1) was significantly influenced by the amount of Smix (A), as its p-value was below the significance threshold. Meanwhile, stirring speed (B) and stirring time (C) showed values close to the significant limit, indicating a potential but weaker effect. To further evaluate the contribution of each factor and quantify effect of different formulation conditions on the transmittance response (Y1), a response surface model was generated using Design Expert software. The final equation of the model describing the percent transmittance can be written as follows:

$$\text{Transmittance (Y1)} = +0.3814 + 48.99\,(A) + 0.2510\,(B) - 0.2473\,(C) - 0.5383\,(AC) + 48.84\,(A^2)$$

Where A is the amount of Smix, B is the stirring speed, and C is the stirring time. Positive values of each factor in the regression equation represent a favorable effect in optimization because they are synergistic, while

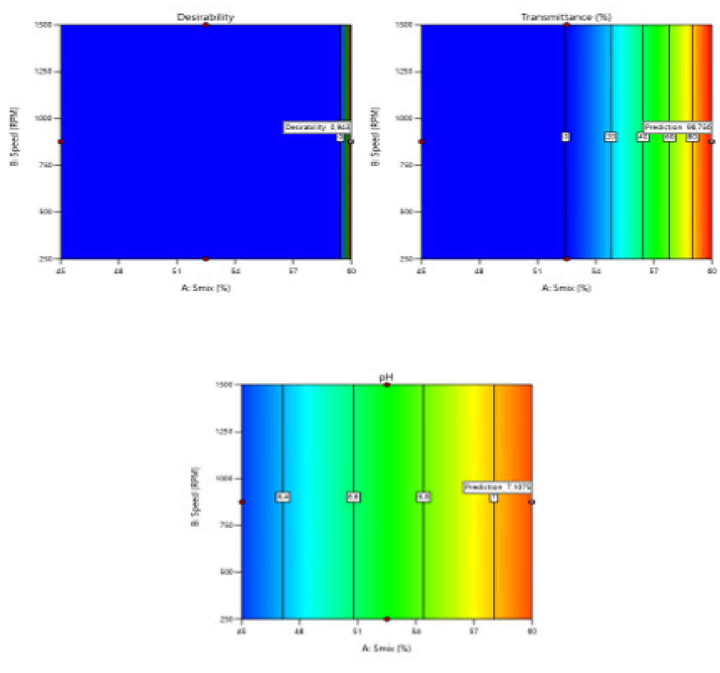


Figure 1. Graphic of nanoemulsion formula optimization results.

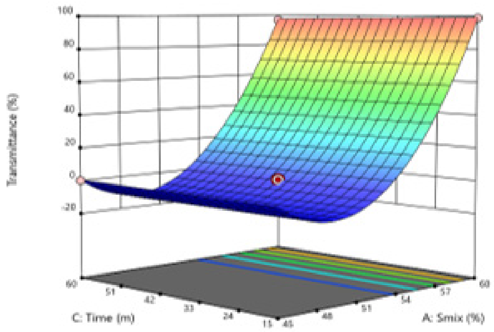


Figure 2. 3D diagram illustrating the effect of nanoemulsion factor on percent transmittance.

negative values indicate an opposite relationship between the factor and the response [17]. Based on the results of the equation for percent transmittance, it was found that the amount of Smix has a synergistic effect or increasing Smix concentration will increase the percent transmittance of the nanoemulsion formula. For visualization of nanoemulsion optimization, the response analysis is plotted in a 3D graphical model in Figure 2.

The amount of Smix has a significant effect on the percent transmittance because it is related to the role of Tween 80 as a surfactant in nanoemulsions. Tween 80 works by emulsifying the oil through adsorption on the surface of oil globules, forming a monolayer, and lowering the interfacial tension of oil and water. Changes in the amount of surfactant will affect the physical characteristics of nanoemulsion, namely percent transmittance. In addition, PEG 400 as a cosurfactant plays a role in stabilizing the globule layer in nanoemulsions, thus keeping the globule diameter constant [18].

The stirring speed (B) and stirring time (C) factors show an insignificant response or no effect in the nanoemulsion formula in the optimization results because the transmittance obtained is in a far range, so the significance value cannot be categorized as a crucial factor. The stirring time determines the duration of interaction between the machine (magnetic stirrer) and the two-phase mixture, which affects the energy and temperature during the nanoemulsion formation process [19]. In addition, sufficient energy is also required in the nanoemulsion formation process, which can be obtained through high-speed stirring to achieve percent transmittance in the 90-99% range [20].

For the pH response (Y2), the P value is smaller than the significance level (0.05), which indicates a significant model where the pH response is influenced by the amount of Smix (A) and stirring time (C), while the stirring speed (B) is not significant so that the pH response is not influenced by the stirring speed in the nanoemulsion manufacturing process. The final equation of the model describing the pH response in the nanoemulsion formula can be written as follows:

pH = + 6.88 + 0.2700 (A) + 0.0175 (B) + 0.1850 (C) - 0.1425 (AC)

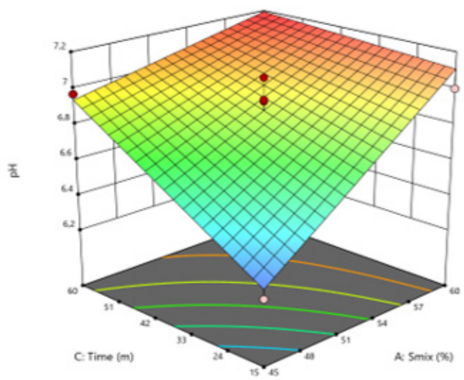


Figure 3. 3d diagram illustrating the effect of nanoemulsion factor on pH.

Table 3. Optimal formula of xanthorrhizol nanoemulsion.

Formula	Smix (%)		Oil Fase (%)		Water (%)	Stirring time (minute)	Stirring speed (rpm)	Average globule size ± SD (nm)	Average polydispersity index ± SD	Average zeta potential ± SD (mV)
	Tween 80	PEG 400	GSO	XNT						
FA	45	15	10	-	30	15	875	14.73333 ± 0.00333	0.07266 ± 0.00002	-70.3666 ± 1.56333
FB	45	15	9.9	0.1	30	15	875	13.76666 ± 0.26333	0.059 ± 0.000097	-48.0333 ± 1.42333

Description:
FA : Nanoemulsion Base Formula
FB : Nanoemulsion Loaded with Xanthorrhizol

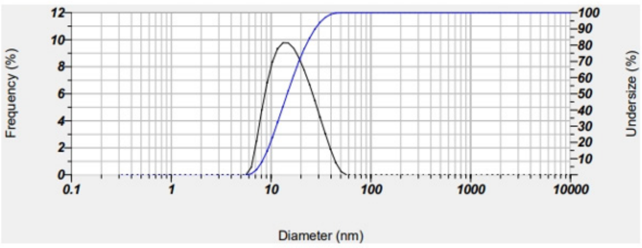
Based on the equation results for the pH response, factors A, B, and C show a synergistic relationship to the pH of the nanoemulsion formula. For visualization of nanoemulsion optimization, the response analysis is plotted in a 3D graphic model in Figure 3.

The pH value of xanthorrhizol nanoemulsion was influenced by the concentration of Tween 80 ($p < 0.05$), where the higher the concentration of Tween 80 added, the higher the pH value of the preparation. Nanoemulsions that use variations of Tween 80 as a surfactant in the formula can produce higher pH values according to the increase in the amount of surfactant. The increase in pH can be attributed to the weakly alkaline nature of the polyoxyethylene chains in Tween 80, which are capable of reducing the availability of free hydrogen ions in the aqueous phase. Additionally, higher surfactant levels enhance interfacial stabilization, reducing the interaction of acidic components with the water phase and contributing to a shift toward a more neutral or slightly alkaline pH [18].

In addition, stirring time showed an effect on the pH of the nanoemulsion, although the change was not statistically significant. This is in accordance with previous research, where the addition of stirring time will tend to reduce the pH value of the preparation [21].

In low-energy emulsification methods, such as spontaneous emulsification, the formation of nano-sized droplets depends on interfacial tension changes facilitated

A



B

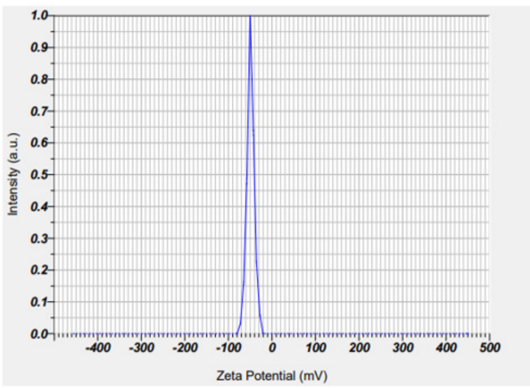


Figure 4. Results of measuring the optimal formula of nanoemulsions xanthorrhizol A. average droplets size and polydispersity index B. Zeta Potential.

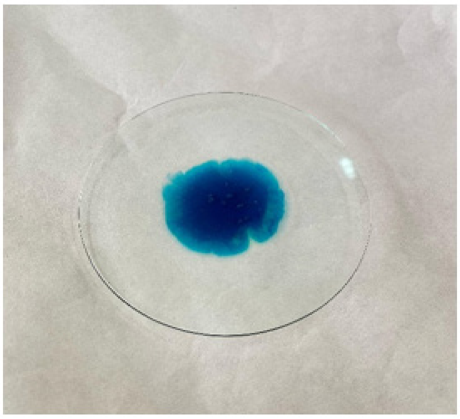


Figure 5. Results of xanthorrhizol nanoemulsion observation using the methylene blue staining method.

by the surfactant, as well as formulation and processing conditions including composition, temperature, and stirring. Stirring plays an important role in promoting interaction between phases and facilitating droplet formation; however, based on the statistical analysis presented, only stirring time showed an observable influence on pH, and the effect was not statistically significant. Stirring speed did not demonstrate an effect on pH. This minor decrease in pH with longer stirring duration is consistent with previous findings, where extended mixing improves homogenization and interfacial stabilization, potentially affecting the distribution of ions within the system rather than altering chemical reactions [22].

The optimum formula was determined through the first derivative of the polynomial equation of transmittance and pH, and the predicted transmittance size and pH were 98.7% and pH 7.1 while the experimental results obtained an average transmittance size and pH of 98.4% and 7.05, resulting in errors of 0.003 and 0.007. The optimization results of the benhken box test method based on the first partial derivative value are the amount of Smix 60%, stirring time 15 minutes, and stirring speed 875 rpm. The formula has a desirability value of 0.943 (close to 1), indicating that the formula is increasingly in accordance with the desired goal [20].

Optimal Formula of Xanthorrhizol Nanoemulsion

The nanoemulsion was successfully formulated according to the optimized composition generated by the Design Expert software. The final formula consisted of Smix, an oil phase (grape seed oil and xanthorrhizol), and water. The oil phase demonstrated good miscibility and stability prior to incorporation with the remaining components. The optimal formula from the Design Expert application can be seen in Table 3.

Xanthorrhizol Nanoemulsion Evaluation Results

Organoleptic Test

Organoleptic testing was conducted on nanoemulsion base formula (FA) and nanoemulsion loaded with xanthorrhizol formula (FB). The test results showed that the nanoemulsion was a clear and homogeneous viscous liquid. The clear color indicates that the particles in the nanoemulsion are nanometer-sized, so light can pass through the solution with minimal resistance or scattering. Homogeneity without phase separation reflects the physical stability and effectiveness of the emulsification process.

Both FA and FB nanoemulsion formulas have a

Table 4. Result of transmittan and pH freeze and thaw stability test.

Cycle	Transmittance			pH		
	1	2	3	1	2	3
0	95.6	96.0	94.9	7.05	7.05	7.28
1	95.1	95.0	95.6	7.15	7.35	7.24
2	95.0	96.3	96.1	7.13	7.16	7.16
3	95.9	95.2	96.9	6.95	6.99	7.04

mild, non-rancid odor indicating that no degradation or contamination has occurred in the formula [23]. There are differences in color and odor in FA and FB, where FB has a bright yellow color and a distinctive odor derived from xanthorrhizol.

Test of Globule Size, Polydispersity Index (PDI), and Zeta Potential

Based on the globule size test results of the nanoemulsion formulas listed in Table 3, formulas FA and FB are included in the nanoemulsion formula value range. In addition, the optimal formula for the nanoemulsion is presented in Figure 4. Smix or a mixture of surfactants and cosurfactants used has an HLB that is able to stabilize the nanoemulsion so as to reduce the globule size. This is consistent with previous studies, where the same use of Smix resulted in small globule sizes ranging from 12 to 14 nm [18].

The polydispersity index shows the level of uniformity of the globule size in the nanoemulsion formula. The measurement results showed that all formulas fell into the nanoemulsion range. PDI values close to 0 indicate homogeneous particle distribution, while values above 0.5 indicate inhomogeneous particles [24]. A PDI value of < 0.5 also indicates that the formula is monodispersed. Monodisperse nanoemulsions with a PDI <0.5 can be obtained through a combination of Tween 80 and PEG 400 which improves the homogeneity of the system and maintains the stability of the formula [25].

Zeta potential is a parameter that controls electrostatic interactions in particle dispersions and can be used to predict the long-term stability of a preparation. Optimal zeta potential values are in the range ≤ -30 mV or ≥ 30 mV, where high negative or large positive charges create repulsive forces between particles thus maintaining dispersion stability [26]. The zeta potential measurement results on the xanthorrhizol nanoemulsion sample showed a value of -48.0333 ± 1.42333 mV. A negative zeta potential value indicates that most of the droplet surface charge is anionic, resulting in a decrease in the droplet surface charge to negative. This value is within the range that indicates good colloidal stability. With this value, the nanoemulsion has a repulsive force strong enough to overcome the forces of attraction between dispersed particles, thus maintaining its stability [26].

pH Test

The degree of acidity (pH) is a value that indicates the acidity or basicity of a substance, solution, or object. The pH test results show that the xanthorrhizol nanoemulsion formula has an average pH value of 7.12 ± 0.017. This is in accordance with the pH range that is safe to apply to the skin, so there is no risk of causing irritation [27].

Percent Transmittance Test

The level of clarity of a nanoemulsion can be determined by the percent transmittance test. The results of the percentage transmittance of the nanoemulsion

Table 5. Comparison of mean transmittance values between freeze–thaw cycles using tukey HSD.

Multiple Comparisons
Dependent Variable: Transmittan
Tukey HSD

(I) Cycle	(J) Cycle	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	1	.26667	.52175	.954	-1.4042	1.9375
	2	-.30000	.52175	.937	-1.9708	1.3708
	3	-.50000	.52175	.776	-2.1708	1.1708
1	0	-.26667	.52175	.954	-1.9375	1.4042
	2	-.56667	.52175	.707	-2.2375	1.1042
	3	-.76667	.52175	.496	-2.4375	.9042
2	0	.30000	.52175	.937	-1.3708	1.9708
	1	.56667	.52175	.707	-1.1042	2.2375
	3	-.20000	.52175	.980	-1.8708	1.4708
3	0	.50000	.52175	.776	-1.1708	2.1708
	1	.76667	.52175	.496	-.9042	2.4375
	2	.20000	.52175	.980	-1.4708	1.8708

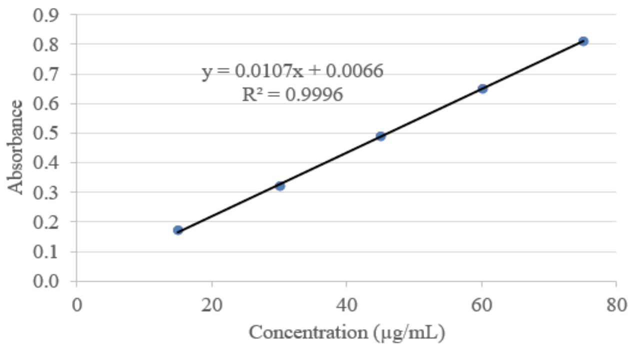


Figure 6. Calibration curve of xanthorrhizol in 96% ethanol.

formula obtained an average value that shows more than 95%. A high percent transmittance value indicates the particle size in the preparation is getting smaller. If the system in the preparation has a very small particle size when light passes through, the light beam will be forwarded, so that the color of the solution in the preparation will look transparent and the percent transmittance value will be greater [28].

Nanoemulsion Type Test

The nanoemulsion type test was carried out by the dilution method, in which the xanthorrhizol nanoemulsion formula was diluted with water in a ratio of 1:100, where the dilution results showed that the preparation could dissolve in water, indicating that the type of preparation formed was oil in water (O/W).

In addition, testing was also carried out by staining method using methylene blue, where methylene blue solution was dripped on the formula placed on a watch glass. The results showed a homogeneous dispersion, which confirmed that the nanoemulsion formed was of the O/W type. The visual result of this evaluation is shown in Figure 5.

Stability Test

The stability test was conducted through two methods, namely centrifugation test and freeze-thaw test. The results of the percent transmittance evaluation (Table 4) did not show signs of instability (<90%) which indicated that the formula was stable to freeze and thaw cycle stability. Evaluation of pH during the freeze and thaw stability test showed a change in pH in the third cycle. Based on the post-hoc Tukey HSD analysis (Table 5 and 6), most pairwise comparisons of pH values between cycles did not show significant differences ($p > 0.05$), indicating that the nanoemulsion maintained relative pH stability during the freeze–thaw process. However, a significant difference was observed between cycle 1 and cycle 3 ($p = 0.029$), suggesting that prolonged exposure to stress conditions may trigger changes in pH. This shift is likely influenced by storage temperature, which may initiate reactions between components in the formulation. Such reactions can lead to either an increase or decrease in pH depending on the chemical characteristics of the ingredients and the applied storage conditions [29].

The centrifugation test results showed that all nanoemulsion formulas had good thermodynamic stability, characterized by no phase separation. The results of this test indicate that all formulas are resistant to the influence of physical forces and can be categorized as stable formulas [26].

Validation of the Analytical Method

The validation parameters evaluated in this study included linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). Linearity testing using five concentration levels (15–75 µg/mL) resulted in a linear regression equation of $y = 0.0107x + 0.0066$ with a correlation coefficient of 0.9997, meeting acceptance criteria and confirming suitability for quantitative analysis. The LOD and LOQ values obtained were 1.642 µg/mL and 5.475 µg/mL, respectively, indicating the method's capability to detect and quantify xanthorrhizol at low concentrations. Accuracy evaluation demonstrated recovery values of 97.4%, 100.1%, and 100.2% at different concentration levels, confirming acceptable accuracy. Precision testing showed a standard deviation of 0.072 and relative standard deviation (RSD) of 0.160%, fulfilling validation requirements and indicating excellent method repeatability.

Table 6. Comparison of mean pH values between freeze–thaw cycles using tukey HSD.

Multiple Comparisons
Dependent Variable: pH
Tukey HSD

(I) Cycle	(J) Cycle	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	1	-.12000	.07071	.384	-.3464	.1064
	2	-.02333	.07071	.987	-.2498	.2031
	3	.13333	.07071	.305	-.0931	.3598
1	0	.12000	.07071	.384	-.1064	.3464
	2	.09667	.07071	.551	-.1298	.3231
	3	.25333*	.07071	.029	.0269	.4798
2	0	.02333	.07071	.987	-.2031	.2498
	1	-.09667	.07071	.551	-.3231	.1298
	3	.15667	.07071	.199	-.0698	.3831
3	0	-.13333	.07071	.305	-.3598	.0931
	1	-.25333*	.07071	.029	-.4798	-.0269
	2	-.15667	.07071	.199	-.3831	.0698

*. The mean difference is significant at the 0.05 level.

Entrapment Efficiency

The entrapment efficiency of xanthorrhizol in nanoemulsion was carried out using a UV-Vis spectrophotometer with a wavelength of 274 nm. Based on the measurement results, it is known that xanthorrhizol nanoemulsion has a relatively low percentage of entrapment efficiency, which is only 58.68% of the total amount of xanthorrhizol. The low amount of xanthorrhizol absorbed in the nanoemulsion system is because the oil phase is quite low in the nanoemulsion formula. According to previous research, a high concentration of oil phase has the ability to absorb more bioactive compounds than oil phase with low concentration [30].

Conclusion

The optimal nanoemulsion formula was obtained based on the BBD method at 60% Smix composition, stirring speed of 875 rpm, and stirring time for 15 minutes. The obtained xanthorrhizol nanoemulsion has characteristics of droplet size 13.7 ± 0.26 nm, polydispersity index 0.059 ± 0.000097, zeta potential -48.03 ± 1.42 mV, transmittance 95.5 ± 0.31%, pH 7.12 ± 0.01, nanoemulsion type is oil in water (O/W), and has a entrapment efficiency value of 58.6%. Xanthorrhizol nanoemulsion formula is stable to freeze and thaw stability test and centrifugation test.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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