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Research Article

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In Vitro Evaluation of Nanoemulsion of Gotu Kola Leaves (*Centella Asiatica* Urb.) as an Alternative Treatment for Tuberculosis

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Abstract

Gotu kola leaves *(Centella asiatica* Urb.) contain asiaticoside compounds that have been demonstrated to possess bactericidal properties against Mycobacterium tuberculosis (*M. tuberculosis),* as evidenced by inhibition tests. Several studies have reported that *M. tuberculosis* has developed resistance to tuberculosis (TB) drugs. The nanoemulsion delivery system represents an innovative therapeutic approach for TB patients, demonstrating significant promise in the diagnosis, treatment, and prevention of infectious diseases such as TB. This study aims to optimize and formulate the manufacture of a gotu kola leaf extract nanoemulsion as an alternative TB treatment. Maceration was employed to obtain a gotu kola leaf extract, yielding 34.7%. Thin-layer chromatography (TLC) analysis revealed a single spot, confirming the presence of asiaticoside. A gotu kola leaf nanoemulsion was developed using the Box-Behnken Design method, incorporating variations in gotu kola leaf extract loading, tween 80, and sonication time. The optimal formula was determined based on particle size parameters, particle size distribution, entrapment efficiency (EE), and extract loading (EL) using Stat-Ease® Design-Expert 360 software. The writing has been corrected. The optimal formula exhibited a particle size of 154.715 nm, a particle size distribution of 0.246, an EE of 92.512%, and an EL of 98.298 mg/g. *In vitro* testing demonstrated the antibacterial potential of the optimum formula, achieving a 56.2% antibacterial effect. In contrast, the positive control rifampicin showed an 89.1% antibacterial effect.

Keywords: Tuberculosis; Gotu kola leaves; Nanoemulsion; *in vitro*.

1. Introduction

Tuberculosis (TB) is a disease caused by infection with the bacteria *Mycobacterium Tuberculosis (M. tuberculosis)*, generally infecting the lungs and can attack other organs in the human body [1]. Based on data from the Global TB Report (GTR) in 2022, Indonesia ranks 2nd in the world for the most TB sufferers with 969 thousand cases. TB cases have increased quite significantly every year, with 274,706 cases recorded in 2022 [2]. One of the main steps to reduce TB cases in Indonesia is improving TB treatment.

Antibiotics are commonly used in TB therapy, but over time, antibiotic resistance has increased. Several studies have reported that *M. tuberculosis* has become resistant to commonly used TB drugs such as isoniazid, rifampicin, ethambutol, streptomycin, ethionamide, and

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kanamycin [3]. Therefore, to reduce antibiotic resistance, it is necessary to explore natural ingredients as safer alternatives to TB therapy.

Gotu kola leaves (*Centella asiatica* Urb.) are abundant natural ingredients found in Indonesia and are known to have antibacterial activity. Research conducted revealed that the asiaticoside compound found in gotu kola possesses bactericidal properties against *Mycobacterium tuberculosis*, as evidenced by the inhibition test. [4]. However, the spread of *M. tuberculosis* bacteria can occur through the air, so testing is high-risk. Therefore, *Bacillus subtilis* is used as an alternative because it has similar DNA base sequences and cell wall structures [5].

The antibacterial properties of gotu kola leaves are attributed to the presence of various compounds, including flavonoids, saponins, tannins, phenols, and steroids [6]. Flavonoids and other phenolic compounds function as antibacterials by forming complexes with extracellular proteins, thereby disrupting the integrity of membranes and cell walls. Saponins, on the other hand, exhibit antibacterial activity by forming complexes with cell membranes via hydrogen bonds, thereby disrupting bacterial cell wall permeability [7, 8, 9].

The triterpenoid component of gotu kola leaves, asiaticoside, employed as a marker compound in this study, exhibits low water solubility, potentially hindering absorption and consequently reducing its bioavailability [10]. Furthermore, asiaticoside's low target tissue absorption is attributed to its substantial molecular weight, a characteristic of the active triterpenoid. To date, research findings have predominantly been presented in extract form. Consequently, asiaticoside was formulated into a nanoemulsion to enhance its absorption and efficacy [11].

The use of nanotechnology is believed to increase the bioavailability of active ingredients due to their small size, thereby increasing their absorption [12]. One of the nanotechnologies used is nanoemulsion. Nanoemulsions can increase the penetration of active ingredients and the stability of active substances, protect against environmental degradation, and improve the absorption of macromolecular compounds due to their small particle size (20-500 nm) [13, 14].

The nanoemulsion oral delivery system represents a groundbreaking innovation in the treatment of tuberculosis. This system delivers targeted, site-specific, and precise drugs using nanotechnology principles. Safety, potency, biocompatibility, biodegradability, non-biotoxicity, and non-immunogenicity are major advantages of nanoemulsion drug delivery. The role of nanoemulsions in improving TB treatment includes short treatment duration, reduced drug side effects, and increased therapeutic efficacy [15].

In addressing the resistance of TB to newly discovered drugs, a comprehensive approach is necessary. While research on new drug delivery systems is crucial at the clinical level, the application of nanotechnology, particularly nanoemulsions, holds significant promise for the diagnosis, treatment, and prevention of infectious diseases such as TB. This study aims to explore the potential of gotu kola leaf extract in the form of nanoemulsion as an alternative treatment for TB.

2. Materials and Methods

2.1. Materials

The tools used were ultrasonic bath (GT Sonic R6 6L; China), oven (Memmert UN 55; Schwabach, Germany), UV-Vis spectrophotometer (Thermo Scientific Genesys-10S; Waltham, MA), centrifugator (Thermo Scientific Heraeus Fresco 17 Centrifuge; Waltham, MA), particle size analyzer SZ-100 (Malvern; United Kingdom), Precisa XB analytical balance (Precisa instruments AG; Dietikon, Switzerland), micropipette (Thermo Scientific; Finnpipette), silica gel F254 Thin Layer Chromatography (TLC) plate, and glassware (Pyrex).

The main ingredient used is gotu kola leaves obtained from farmers (Tawangmangu, Indonesia), which have been identified and authenticated by Setia Budi University (Surakarta, Indonesia). The formulation ingredients consisted of tween 80 and propylene glycol from Merck (Darmstadt, Germany), and oleic acid from Sigma-Aldrich (St. Louis, MO). Pro analysis chemicals: asiaticoside standard (Sigma-Aldrich; USA) and pro analysis purity chloroform were purchased from Merck (Darmstadt, Germany). Other materials used were 70% ethanol, methanol, and Aquadest obtained from a local supplier (Agung Jaya, Surakarta, Indonesia). *Bacillus subtilis* ATCC No. 6633 bacteria were obtained from the Microbiology Laboratory, Faculty of Medicine, UNS. Nutrient agar (NA) and nutrient broth (NB) media were obtained from Merck (Darmstadt, Germany), and rifampicin was obtained from Tianjin Pharm (China).

2.2. Extraction and Purification of Gotu Kola Leaves (Centella asiatica Urb.)

Fresh gotu kola leaves were washed and dried in an oven at 40°C for 24 hours. Gotu kola leaves were ground, weighed up to 500 g, macerated in 70% ethanol for 24 hours, and evaporated to obtain a thick extract. The yield was calculated from the resulting thick extract with the following calculation (Equation 1):

$$\%\text{Yield} = \frac{\text{Weight extract obtained (gram)}}{\text{Simplified weight before extraction (gram)}} \text{X } 100\% \quad (1)$$

2.3. Identification and Quantification of Total Asiaticoside Levels

Qualitative identification of asiaticoside using the thin-layer chromatography (TLC) method. Gotu kola leaf extract and the reference were spotted on a silica gel GF254 plate as the stationary phase. The mobile phase used was chloroform: methanol: aquadest at 64:50:10. Elution was carried out after the mobile phase was saturated; the plate was then dried and visualized under UV light at 254 and 366 nm. The Rf value can be calculated using the following formula (Equation 2):

$$\text{Rf} = \frac{\text{The distance the compound is moved from the point of origin}}{\text{The distance the solvent moves from the origin}}. \quad (2)$$

A total of 50 mg of pure asiaticoside standard was weighed and dissolved in 50 ml of Aquadest to obtain a 1000 ppm solution. Sample solutions of 10, 20, 30, 40, 50, and 60 ppm were prepared by pipetting 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6, respectively. Each ppm was pipetted into 10 ml of Aquadest, and the solution was read at 209 nm [16]. Weighing 50 mg of gotu kola leaves extract and dissolving it in 50 ml of Aquadest produced a sample solution with a concentration of 1000 ppm. The sample solution was prepared at a concentration of 40 ppm, and its absorbance was measured at the maximum wavelength of asiaticoside, 209 nm. The percentage of asiaticoside in gotu kola leaf extract was calculated using the asiaticoside standard curve.

2.4. Formulation of Nanoemulsion from Gotu Kola Leaves Extract

Gotu kola leaf extract was dissolved in 70% ethanol, then mixed with oleic acid until homogeneity was achieved using a magnetic stirrer (1000 rpm). Subsequently, tween 80 and propylene glycol were mixed with a small amount of Aquadest until homogeneity was attained, representing the water phase. Subsequently, the oil phase was gradually added to the water phase while stirring for 10 minutes. Finally, aquadest was gradually added, and the stirring speed was increased to 1250 rpm for another 10 minutes [17]. All the mixed ingredients were then homogenized using an ultrasonicator for 30 minutes. Furthermore, the finished preparation was put into a 10 mL vial.

2.5. Characterization of Gotu Kola Leaves Extract Nanoemulsion

Particle size characterization was performed by centrifuging 2 mL of each nanoemulsion at 5,000 rpm for 5 minutes. The supernatant was diluted to 1 mL with 10 mL of distilled water, and particle size testing was carried out using a particle size analyzer (Malvern, UK) based on dynamic light scattering at 209 nm, yielding the particle size distribution. Determination of entrapment efficiency (EE) and extract loading (EL) was performed by the complexometric method using a UV-Vis spectrophotometer. Each formula was diluted to 2 mL and centrifuged for 10 minutes at 5000 rpm. 1 mL of supernatant was pipetted and diluted with distilled water to the boundary mark in a 10 mL measuring flask. The solution was read at λmax 209 nm until the sample absorbance fell within the calibration curve range. EE was calculated as a percentage of the absorbed drug content to the actual drug content (Equation 3). The EE results can then be used to calculate the EL value (Equation 4) [18].

$$\%\text{EE} = \frac{\text{Drug in system} - \text{over-the-counter drug}}{\text{Drug in the system}} \times 100\% \quad (3)$$

$$\text{EL (mg/g)} = \frac{\text{Observed drug weight}}{\text{Total medicine in the system}} \quad (4)$$

2.6. Experimental Design of Nanoemulsion Formulation

The development of a gotu kola leaf extract nanoemulsion was conducted using a Box-Behnken design to obtain 15 formulations with extract loading, tween 80 concentration, and sonication time as variables. These parameters will determine the critical characteristics of the nanoemulsion, namely particle size (PS), polydispersity index (PDI), entrapment efficiency (EE), and extract loading (EL). The data were analyzed using multiple linear regression

(Equation 5), and contour plots were generated from the equations for each response.

$$Y = \beta + a\times A + b\times B + c\times C + ab\times AB + ac\times AC + bc\times BC + a\times A2 + b\times B2 + c\times C2 \quad (5)$$

2.7. Optimization of Formula Composition

The parameters of particle size (PS), polydispersity index (PDI), entrapment efficiency (EE), and extract loading (EL) were analyzed using StatEase® 360 Design-Expert software (Minneapolis, MN) employing a Box-Behnken design and ANOVA with a quadratic model until a contour plot response was obtained. The four generated contour plots were subsequently used to determine the optimal formula area using the superimposed contour plot technique [19, 20].

2.8. In-Vitro Antibacterial Activity Test

The method employed is the 5+1 technique, which involves utilizing five distinct standard concentrations and one sample concentration. A *Bacillus subtilis* bacterial suspension was prepared in *Nutrient Broth (NB)* media and diluted to approximately 700 µL. Subsequently, the suspension was spread onto the surface of NA media using a Drigalsky instrument. A disc paper containing the drug and standard was then placed on top of the media. The incubation process was conducted for 24 hours at 37°C. The positive control used was rifampicin. Subsequently, observations were conducted by measuring the inhibition zone diameter with a caliper and calculating the percentage of antibiotic potential [21, 22].

3. Results and Discussion

3.1. Centella asiatica Leaves Extract

Determination of gotu kola leaves simplicia, which has been carried out to identify and quantify gotu kola leaves simplicia, has obtained the following results:

Kingdom: Plantae; Super Division: Spermatophyta; Division: Magnoliophyta; Class: Magnoliopsida; Order: Umbellales; Family: Umbelliferae; Genus: Centella; Species: Centella asiatica Urb. Determination Results according to Steenis, CGGJV, Bloembergen, H, Eyma, PJ 1992: 1b–2b–3b–4b–6b–7b–9b–10a. Group 7. 92b–100b–103b–105b–106b–107b–108b. family 98. Umbelliferae. 1b–2b–3. Centella. *Centella asiatica* Urb.

Wet sorting was carried out on the samples to remove dirt still attached to the gotu kola leaves simplicia. From 500 grams of gotu kola leaves simplicia, the extract obtained was 101.38 grams, yielding 20.28%, with a dark green to blackish color and a distinctive odor of gotu kola leaves (**Figure 1**).

3.2. Identification and Quantification of Total Asiaticoside Levels

Identification of asiaticoside compounds using Thin Layer Chromatography (TLC) is a qualitative method. The TLC is performed to identify the presence of asiaticoside compounds in the gotu kola leaf extract. The principle of TLC is to separate compounds based on their absorption and partitioning, influenced by the stationary and mobile phases. The stationary phase is a TLC plate of silica gel GF254, which is polar, and the mobile phase is chloroform:methanol:aquadest (64:50:10).

Figure 1. Results of the extraction of gotu kola leaves (*Centela asiatica*) using the maceration method for 1x24 hours with 70% ethanol solvent.

Observation under UV light at 254 nm showed a fluorescent plate, while the sample remained dark (**Figure 2a**). Observation under UV 366 nm showed fluorescent spots and a dark plate (**Figure 2b**). Elution of the asiaticoside standard (s) and the test sample (u) showed almost the same elution distance. After calculating the Rf, the Rf value for the asiaticoside standard(s) was 0.975, and the Rf value for the test sample (u) was 0.9875. The results obtained indicate that the gotu kola leaf extract contains the active antibacterial compound asiaticoside.

Asiaticoside levels in gotu kola leaf extract were quantified using a UV-Vis spectrophotometer. The wavelength used was 209 nm, which is the maximum wavelength of asiaticoside. The calibration curve was y = 0.0099x + 0.2467, with an R^2 of 0.9933. From the equation above, the results were 34.7%, indicating that the asiaticoside content meets the requirements, as stated in Farmakope Herbal Indonesia, which specifies asiaticoside content of not less than 0.07% [23].

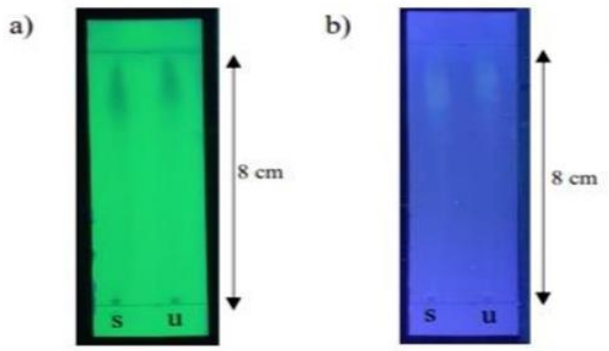

Figure 2. Results of TLC at wavelengths of 254 nm (a) and 366 nm (b)

3.3. Evaluation and Characterization of Nanoemulsion Preparations Based on Design Expert

Based on the 15 suggested formulas (**Table 1**), the nanoemulsion obtained was a greenish-white emulsion, stable and foggy in the long term, and no sediment was formed during 5 days of storage at 25°C. Statistical analysis of the nanoemulsion formula was carried out by regressing the selected response surface to estimate the coefficients and generate a contour plot. The contour plot analysis obtained was used to optimize and determine the interaction coefficient between the various factors. The results of the ANOVA test with a confidence level of 95% ($p < 0.05$) for each response are shown in **Table 2**.

The results of the particle size response (PS) obtained sizes in the range of 137.14 - 179.79 nm. Based on the PS response (**Figure 3a**), the lower the extract loading and the higher the tween 80 concentration, the greater the PS value. The concentration of Tween 80 significantly affects the nanoemulsion's size. As a surfactant, Tween 80 can reduce surface tension, resulting in a smaller droplet size [24]. The interaction between extract loading and sonication time (**Figure 3b**) also shows that, for lower extract loading and longer sonication time, the PS value is higher. Research indicates that particles at the nanoscale can enhance permeability through capillary walls and extend drug clearance [25]. The higher the Tween 80 concentration and the longer the sonication time, the greater the PS value obtained (**Figure 3c**). The results of the analysis of particle size values are explained by multiple linear regression analysis in Equation 6.

PS (nm) = 152,286.38A+ 10.81B+3.86C+ 3.09AB+ 1.99AC+ 8.40BC+ 1.9A2+ 3.16B2+ 1.11C2 (6)

Equation 6 shows that individually varying the loading of extract, tween 80, and sonication time can increase particle size. The interaction of each factor also shows that each interaction can increase particle size. In this equation, it can be concluded that increasing the concentration of Tween 80 plays a dominant role in influencing particle size in this model.

The results of the polydispersity index (PDI) response were in the range of 0.235-0.388, indicating that the nanoemulsion preparation in this study had a uniform particle size distribution, as evidenced by the PDI value below 0.5 [26]. Based on the results of the PDI response (**Figure 4a, 4b**), the lower the concentration of gotu kola leaf extract with tween 80 and the faster the sonication time, the lower the PDI value. The interaction between sonication time and Tween 80 concentration (**Figure 4c**) shows that the faster the sonication time, the lower the resulting PDI value. It demonstrated that a shorter sonication duration results in a smaller particle size [27]. The multiple linear regression model explains the results of the PDI analysis in Equation 7.

PDI102=0.321+ 0.0245A- 0.0259B- 0.0006C-0.0227AB+ 0.0203AC- 0.008BC+ 0.0458A2+ 0.0275B2+ 0.0255C2 (7)

Table 1. The nanoemulsion formulation of gotu kola leaf extract was optimized using a Box–Behnken Design, with some components held constant.

Factor	Level		
	-1	0	+1
A (%)	80	85	90
B (%)	2	2,5	3
C (minutes)	3	6	9
Constant components of nanoemulsion			
Oleic acid	ad 100% in the oil phase		
Propylene Glycol	at a ratio of 9:1 with Tween 80		
Aquadest	ad 100% in the water phase		

Notes: The ratio of the oil phase was 10%, and the water phase was 90%. The components that varied were gotu kola leaf extract (%), Tween 80 (%), and sonication time (minutes), which were designed using a design of experiments (DoE) approach in Stat-Ease® 360 Design Expert software with the Box-Behnken Design model to produce 15 formulas.

Table 2. Characterization results of the gotu kola leaves extract nanoemulsion preparations on several parameters

Parameters	Y1 (nm)		Y2		Y3 (%)		Y4 (mg/g)	
	CR	p	CR	p	CR	p	CR	p
Intercept	152.28	-	0.321	-	47.05	-	96.39	-
A	-6.38	0.0001*	-0.025	0.1271	2.07	0.0429*	0.833	0.0013*
B	10.81	0.0001*	-0.026	0.1114	6.57	0.0004*	0.396	0.0270*
C	3.86	0.0001*	-0.001	0.9646	5.06	0.0012*	0.175	0.2295
AB	3.09	0.0001*	-0.023	0.2838	0.86	0.4671	0.172	0.3856
AC	1.99	0.0001*	0.020	0.3342	27.4	0.0001*	1.74	0.0002*
BC	8.40	0.0001*	-0.008	0.6905	-6.46	0.0019*	-0.175	0.3781
A^2	1.90	0.0001*	0.046	0.0681	-5.57	0.0044*	-0.722	0.0121*
B^2	3.16	0.0001*	-0.028	0.2221	16.70	0.0001*	0.950	0.0039*
C^2	1.11	0.0001*	-0.026	0.2527	22.58	0.0001	0.625	0.0210*
P	0.0001		0.2032		0.0001		0.0016	
R^2	0.9999		0.7964		0.9965		0.9759	
Adj R^2	0.9998		0.4300		0.9902		0.9325	
Pred R^2	0.9989		0.5249		0.9440		0.6146	
AP	331.9551		4.9470		36.5594		17.4400	

Notes: *$p < 0.05$ (significantly different); A = Extract *loading* (%); B = Tween 80 (%); C = Sonication time (min); CR = regression coefficient; *p, p-value*; Y1 = Particle size (nm), Y2 = Polydispersity index; Y3 = *Entrapment efficiency* (%); Y4 = *Drug Loading* (mg/g); R^2 = coefficient of determination; Adj. R^2 = *Adjusted* R^2 ; Pred R^2 = *Predicted* R^2 ; AP = *adequate precision*

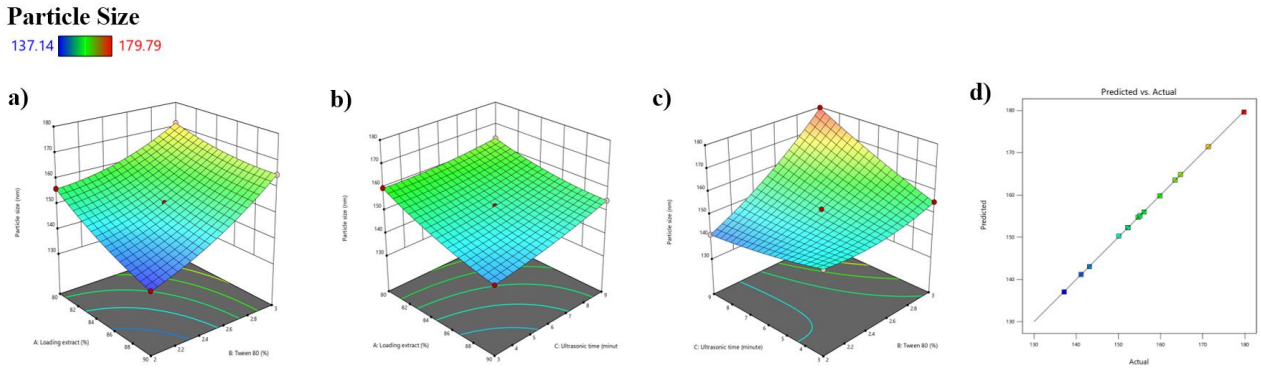

Figure 3. Contour plot of particle size (PS) at maximum interaction between extract loading and tween 80 (a), interaction between extract loading and ultrasonic time (b), interaction between tween 80 and sonication time (c), and quadratic PS model.

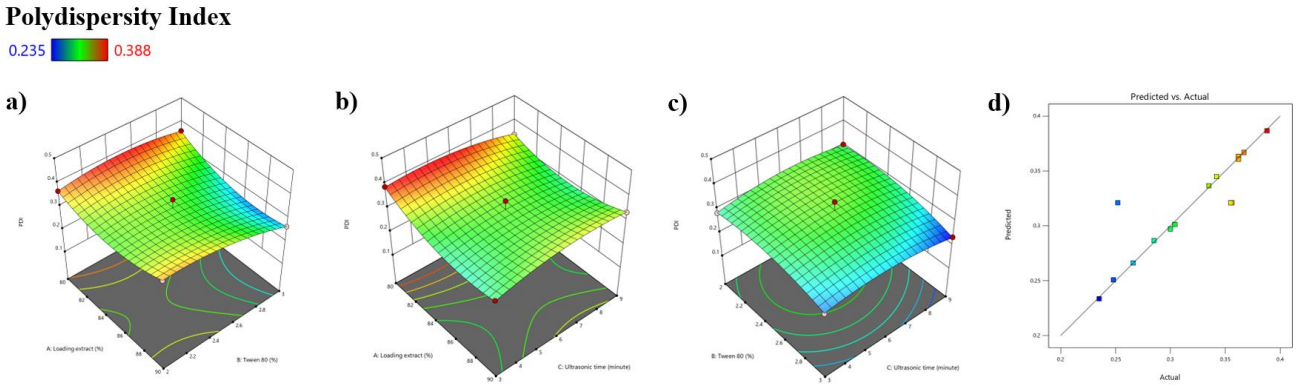

Figure 4. Contour plot of polydispersity index (PDI) at maximum interaction between extract loading and tween 80 (a), interaction between extract loading and sonication time (b), interaction between tween 80 and sonication time (c), and quadratic PS model.

Equation 7 shows that individually extracting the loading and sonication time can increase the PDI value. However, the interaction between extract loading and Tween 80 in the system decreases the PDI value. The interaction between tween 80 concentration and sonication time in the system decreases the PDI value. From this equation, it can be concluded that increasing the extract loading and the Tween 80 concentration play a dominant role in influencing the PDI value.

The entrapment efficiency (EE) response ranged from 33.616% to 96.167%. The EE response results (**Figure 5a**) show that higher extract loading and tween 80 increase the EE value. The interaction between low extract loading and longer sonication times (**Figure 5b**) will yield higher EE values. Prolonged sonication can elevate high extracellular enzyme (EE) levels and disrupt the lipid bilayer [28]. The interaction between Tween 80 concentration and longer sonication times can reduce the EE value (**Figure 5c**). This is supported by research demonstrating a significant correlation between the concentration of tween 80 and the enhancement of EE values [29]. The multiple linear regression model explains the results of the EE value analysis in Equation 8.

$$EE\%=47.05+2.07A+6.57B+5.06C+0.8555AB+27.4AC-6.46BC-5.57A2+16.7B2+22.58C2 \quad (8)$$

Equation 8 shows that, individually, the concentration of Tween 80 and the sonication time can reduce the EE value. However, individually, the loading of the extract and Tween 80 can increase the EE value. The interaction between the loading of the extract and sonication time in the system can increase the EE value. From this equation, it can be concluded that increases in the concentration of tween 80 and sonication time play a dominant role in influencing the EE value.

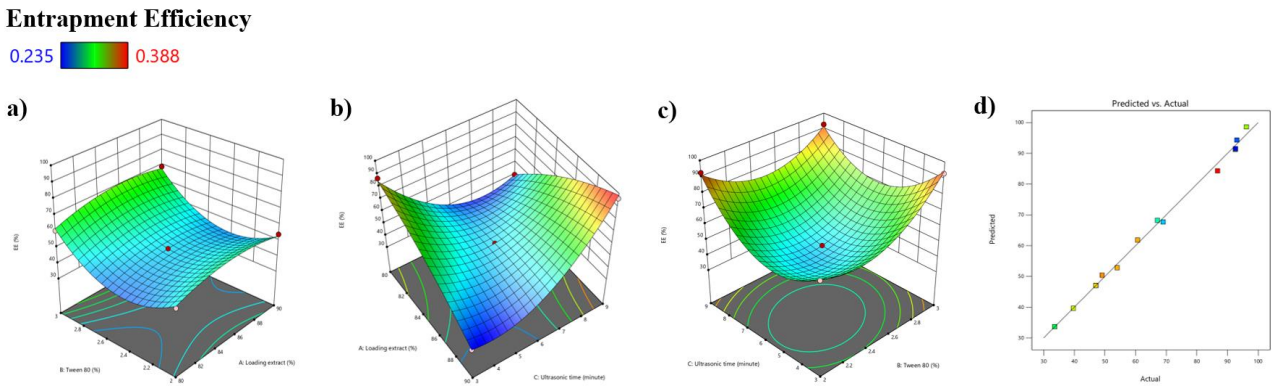

Figure 5. Contour plot of entrapment efficiency (EE) at maximum interaction between extract loading and tween 80 (a), interaction between extract loading and ultrasonic time (b), interaction between tween 80 and sonication time (c), and quadratic PS model.

The extract loading (EL) response ranged from 94.075 to 98.858 mg/g. Based on the EL response results (**Figure 6a**), the higher the extract loading, the greater the EL value. Tween 80 also increases the EL value.

It has been reported that components of Tween 80 with varying chain lengths can increase EL levels due to differences in the extent of hydrocarbon interactions [26]. **Figure 6b** shows that, for a given extract loading, the longer the sonication time, the higher the EL value. The higher the Tween 80 concentration and the longer the sonication time, the higher the EL value will be (**Figure 6c**). The multiple linear regression model explains the results of the EL value analysis in Equation 9.

$$EL=96.39+0.8329A+0.3957B+0.1749C+0.1718AB+1.74AC-0.1747BC-0.7222A2+0.9495B2+0.6247C2 \quad (9)$$

Equation 9 shows that individually loading the extract with Tween 80 and sonication time can increase the EL value. However, individually, sonication time can decrease the EL value. The interaction between Tween 80 concentration and sonication time can decrease the EL value. From this equation, it can be concluded that increasing the concentration of Tween 80 and the sonication time play a dominant role in influencing the EL value.

3.4. Optimization of Gotu Kola Leaves Extract Nanoemulsion Formula

The optimum formula was obtained based on the overlay plot (**Figure 7a**), which combines several contour plots to obtain the optimum area, then based on the desirability value, the most optimum formula was obtained, namely with a combination of 85.36% gotu kola leaf extract loading, 3% tween 80, and 15 minutes of sonication time. Macroscopic visualization (**Figure 7b**) was performed to evaluate and justify the formation of nanoemulsions qualitatively.

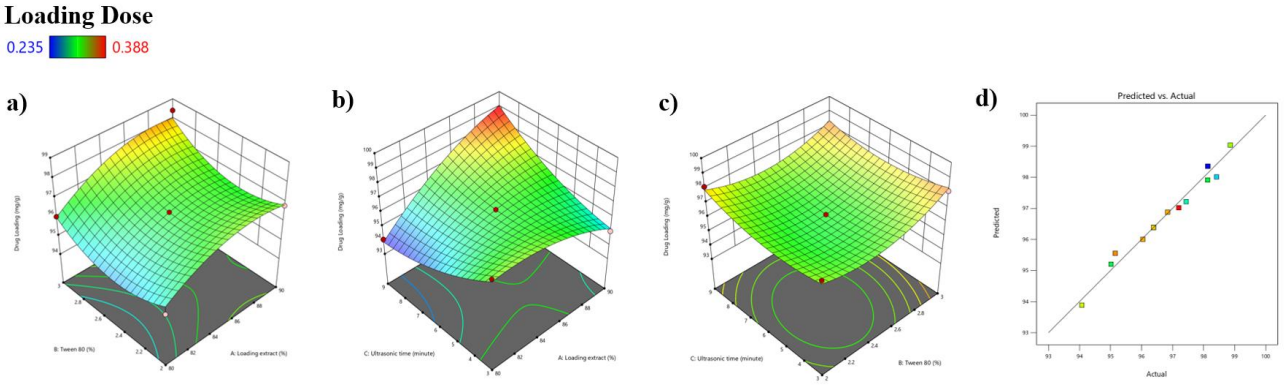

Figure 6. Contour plot of extract loading (EL) at maximum interaction between extract loading and tween 80 (a), interaction between extract loading and sonication time (b), interaction between tween 80 and sonication time (c), and quadratic model of EL.

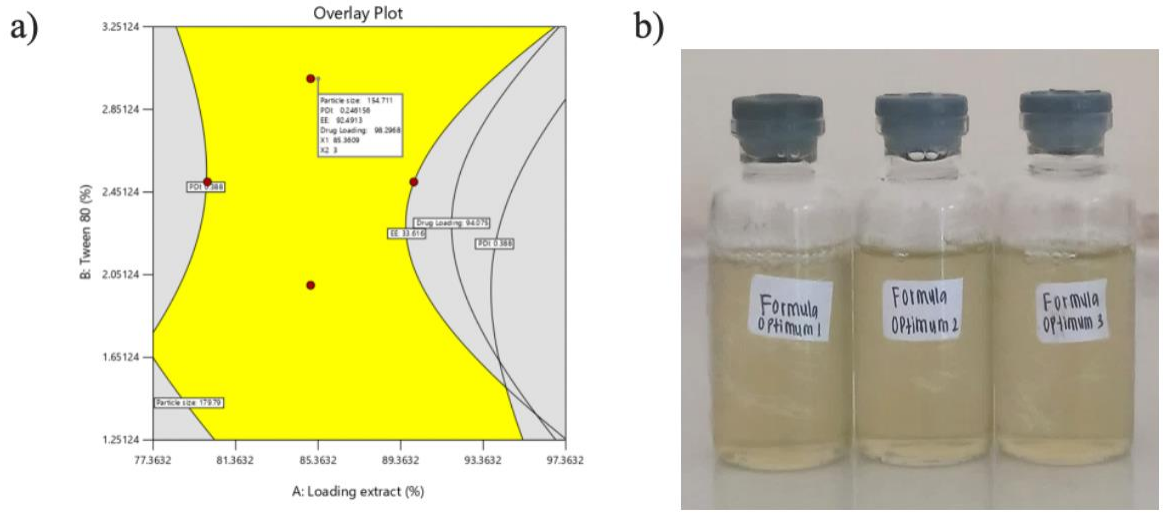

Figure 7. Overlay plot of optimum nanoemulsion formula (a); visualization of nanoemulsion in macroscopic(b)

3.5. In-Vitro Antibacterial Effectiveness

This test was conducted using the agar diffusion method with a 5+1 design, in which five levels of reference solution concentration were used, with the middle level as the reference, and 1 sample solution at each concentration level. The sample concentration used must be within the standard concentration range used with a concept such as determining levels with a calibration curve. The advantage of the 5+1 method over methods such as 1+1 or 3+3 is that it calculates results by accounting for the diameter of the inhibition correction in the standard/positive control, thereby yielding more accurate results [30].

The design components used, consisting of S1, S2, S3, S4, and S5, are the concentrations of the comparison solution at different dosage levels, in sequence (**Figure 8**). Component S3 is the comparison solution with the middle dose. The results are presented in **Table 3**. From these results, a calibration curve is generated to estimate the antibacterial potential of the sample.

Based on the data, a linear regression equation was obtained: $y = 6.4209x + 12.154$, with an R^2 value of 0.9857. The R^2 value is 0.9857, indicating that the calibration curve is linear. This is because the R2 value is close to 1. The linear relationship between the log of the antibiotic dose and the diameter of the inhibition zone is depicted in the graph. This means that the more antibiotics are consumed, the wider the inhibition zone produced.

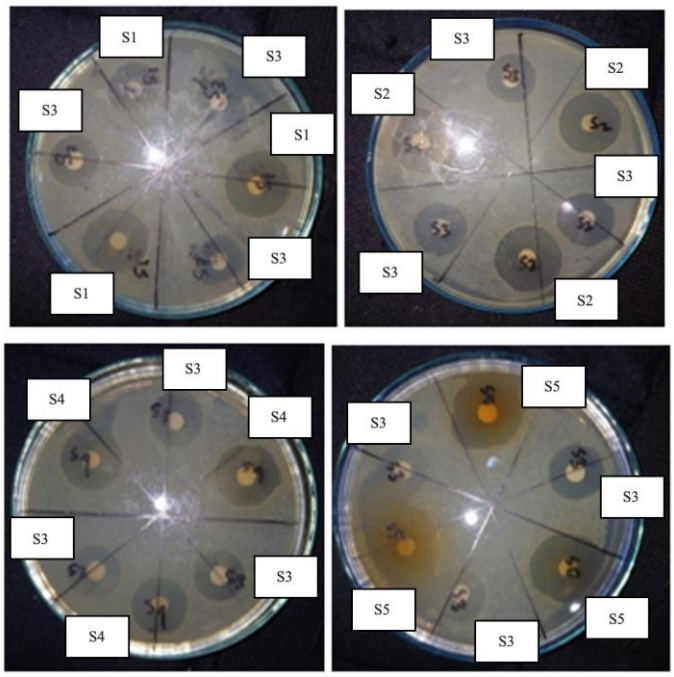

Figure 8. Results of the blandness of S1, S2, S3, S4, and S5.

Table 3. Presents data on rifampicin dose logarithms and corrected inhibition zone diameters. It also includes linear regression calculations (X^2, Y^2, and XY) for analyzing the dose-effectiveness relationship.

	Dose	Dose Log (X)	Inhibition Zone Diameter (Y)	X2	Y2	XY
S1	1.60	0.20	13.38	0.04	179.11	2.73
S2	2.00	0.30	14.22	0.09	202.27	4.28
S3	2.50	0.40	14.61	0.16	213.40	5.81
S4	3.13	0.49	15.46	0.24	238.87	7.65
S5	3.91	0.59	15.88	0.35	252.10	9.40

Description: Log Dose (x)=Logarithm of the standard antibiotic concentration (rifampicin); Correction Result= Corrected Average Inhibition zone of each rifampicin concentration; XY= Calculation of the Consequences of X and Y.

Three samples were used (**Figure 9**): the optimum formula drug sample (U1), the gotu kola leaf extract sample (U2), and the positive control sample of the antibiotic rifampicin (U3). After incubation for 24 hours, the diameter of the inhibition zone was measured and the percentage of antibiotic potential was calculated and the results of the antibiotic potential for the optimum formula nanoemulsion were 56.2%; the results of the antibiotic potential for the gotu kola leaf extract sample were 59.6%; and the results obtained in the positive control of the antibiotic rifampicin were 89.1%.

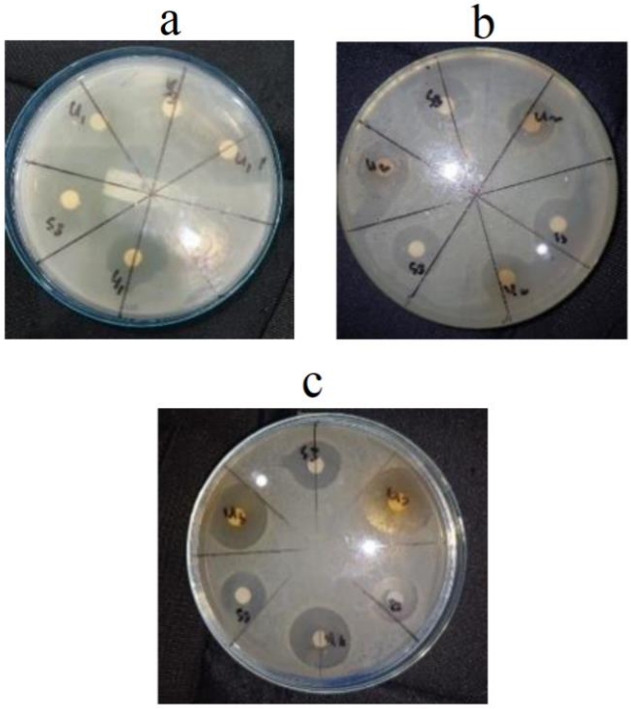

Figure 9. Antibacterial activity test on optimum formula drug samples (a) Nanoemulsion preparation of gotu kola leaf extract, (b) Test on gotu kola leaf extract samples, (c) Positive control activity test for rifampicin antibiotics

From the data in **Table 4**, calculations are carried out to obtain the standard deviation (SD) and relative standard deviation (RSD) values. Standard deviation is a statistical measure that indicates how far the data are spread from their average. From these results, the consistency of the measurement results in the bland zone across several samples is indicated. A smaller value means a more consistent result than a larger value. The relative standard deviation measures the variability of the data relative to the average size and helps assess the consistency of antibacterial effectiveness across various samples. The results obtained were compared between U1 and U3; the U2 value was 0.05, the smallest value (**Table 4**). The results show that the U2 value is more consistent than U1 and U3. The RSD value is considered good if it is below 5%, indicating that the data obtained is precise. The results from the three samples showed that all were good, as the samples had values of less than 3%. The RSD value for sample U2 was 0%, indicating that the results were good and very precise, with no significant variation.

Table 4. Presents the results of the precision test on the inhibition zone diameter of various samples, showing average and RSD for consistent measurement.

Sample	Resistance Diameter Average	RSD
U1	14.15 ± 0.362	3%
U2	14.86 ± 0.050	0.3%
U3	19.74 ± 0.396	2%

RSD= Relative Standard Deviation

Based on the results presented in **Figure 9**, the optimal sample for the nanoemulsion preparation of gotu kola leaf extract (U1) showed a potential antibacterial activity of 56.2%. In contrast, the pure gotu kola leaf extract (U2) showed a slightly higher antibacterial activity, reaching 59.6%. For comparative purposes, the positive control sample, formulated with the antibiotic rifampicin (U3), exhibited the highest antibacterial activity, with a percentage of 89.1%. The antibacterial potential data obtained indicate a decline in antibacterial activity compared to the optimal nanoemulsion of gotu kola leaf extract. While the nanoemulsion formulation retains some antibacterial activity, further improvements are needed to enhance its effectiveness and achieve optimal therapeutic results.

Gotu kola leaf extract contains bioactive compounds, such as flavonoids and saponins, known for their antibacterial properties. However, extraction and formulation as a nanoemulsion affect compound stability, as do pH, temperature, and other ingredients. Research shows that the active compounds in plant extracts degrade under environmental conditions, reducing their antibacterial potential. An inappropriate combination of active ingredients in pharmaceutical formulations can reduce antibacterial activity, especially when one component inhibits another. The potency of the optimum formulation decreases, but it's not significantly different from that of pure gotu kola leaf extract. Gotu kola leaves show potential as an alternative treatment to rifampicin for TB, especially due to drug resistance. Though less effective than rifampicin, gotu kola leaves may be safer and more sustainable in the long term. The main reason for considering them as an alternative is the issue of rifampicin resistance. Gotu kola leaves offer several benefits compared to conventional drug preparations. They protect the liver from damage caused by synthetic drugs and enhance the body's immune response in TB patients. A strong immune system helps fight infections more effectively, reducing the need for high-dose drugs with side effects. This improves patient compliance and provides additional benefits in TB therapy.

4. Conclusion

A nanoemulsion formulation of gotu kola leaf extract was successfully developed using the Box-Behnken design technique. This resulted in an optimal formula with a gotu kola leaf extract concentration of 85.36%, tween 80 at 3%, and a sonication time of 15 minutes. The antibacterial potential of the optimum formula was 56.2%; the potential of the gotu kola leaf extract sample was 59.6%; and the positive control rifampicin was 89.1%. This indicates that the antibacterial activity of the nanoemulsion formulation from gotu kola leaf extract remains lower than that of rifampicin.

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Conflict of interest

The authors declare no conflicts of interest.

Data availability

This study generated or analyzed data as described in the manuscript. All information presented in this article is readily available for access.

Authors Contributions

S. Faazatus: conceptualization of the idea, formulation of research data, writing the initial draft; SR Ningsih: methodology design, software development; A. Haifani: verification, data collection/research evidence; N. Herawardhani: data presentation, editing; A. Ainurofiq: supervision, validation, and reviewing the publication.

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Using artificial intelligence chatbots

There was no use of artificial intelligence in the making of this article.

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