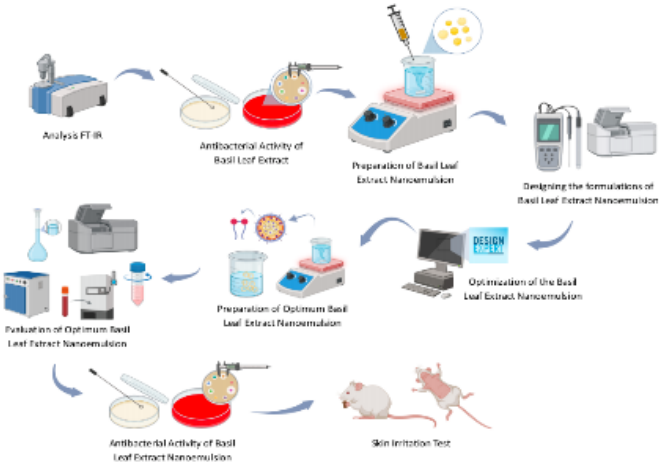
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Optimization and Formulation of Nanoemulsion Extracts Basil Leaves (*Ocimum basilicum* L.) and Testing of Anti-Acne Activity against *Propionibacterium acnes*

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Abstract: Acne vulgaris is a common dermatological condition that is often worsened by hot and humid climates, such as those in Indonesia. These conditions promote the growth of *Propionibacterium acnes*, a bacterium involved in acne pathogenesis. Long-term use of topical antibiotics may lead to resistance, highlighting the need for natural alternatives. This study aimed to formulate and optimize a nanoemulsion of basil leaf extract (*Ocimum basilicum* L.) and evaluate its antibacterial activity against *P. acnes*. The nanoemulsion was prepared by spontaneous emulsification using Tween 80 as the surfactant and PEG 400 as the co-surfactant. Optimization was performed using the Box-Behnken Design with Design Expert 13 software, considering pH and transmittance as responses. The optimal formulation contained Tween 80 (41.09 %) and PEG 400 (23.35 %), resulting in a nanoemulsion with a particle size of 13.80 ± 0.36 nm, PDI 0.18 ± 0.04, and zeta potential –20.00 ± 0.80 mV. The formulation showed good physical stability and caused no irritation in animal skin tests. Antibacterial testing showed an inhibition zone diameter of 12.30 ± 0.28 mm, indicating weak activity. These findings suggest that basil leaf extract nanoemulsion has potential as a natural topical alternative for acne treatment.



Keywords: *Ocimum basilicum* L, *Propionibacterium acnes,* nanoemulsion, antibacterial, Box-Behnken Design.

1 Introduction

In Indonesia, a tropical country with a year-round hot and humid climate, the proliferation of skin microorganisms such as *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* is facilitated. These microorganisms play a pivotal role in the pathogenesis of acne [1)]. In clinical practice, the treatment of moderate to severe acne

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typically involves a combination of topical retinoids, benzoyl peroxide (BPO), and topical antibiotics such as erythromycin or clindamycin [2]. Nevertheless, the efficacy of antibiotic therapy is increasingly jeopardized by the proliferation of resistance, largely attributable to *P. acnes* capacity to form biofilms. Biofilms composed of polysaccharide matrices, proteins, and DNA have been shown to impede antibiotic penetration and facilitate bacterial resistance [3]. The resistance rate of *P. acnes* to erythromycin and clindamycin in Indonesia has been documented to reach 57.7 % [4], underscoring the urgent need for alternative approaches.

One such innovative approach that has been developed is a nanoemulsion-based delivery system. Nanoemulsions are colloidal dispersion systems with droplet sizes ranging from 10 to 200 nanometers. These systems have been shown to enhance the solubility of hydrophobic compounds, improve the penetration of active substances through the skin, and exhibit significant antibiofilm activity [5,6]. In the process of forming nanoemulsions, the selection of surfactants and co-surfactants exerts a significant influence on the characteristics of the resulting system. Tween 80, a non-ionic surfactant, has been demonstrated to be safe and capable of reducing interfacial tension, while PEG 400, functioning as a co-surfactant, has been shown to enhance system stability through the formation of intermolecular bonds [7,8]. The combination of both has been widely used and proven to produce a nanoemulsion system with small droplets and uniform distribution [9].

Nanoemulsion systems hold considerable promise for the encapsulation of bioactive compounds derived from herbal plants, such as basil leaves (*Ocimum basilicum* L.). Ethanol extracts of basil leaves have been found to contain a variety of active compounds, including flavonoids, saponins, essential oils, tannins, phenolics, terpenoids, and alkaloids. These compounds have demonstrated antibacterial activity [10]. Flavonoids function by disrupting bacterial cell membrane permeability, inducing cellular leakage and subsequent cell death [11]. A multitude of studies have demonstrated the antibacterial properties of basil leaf extract against *P. acnes*, *S. aureus*, *E. coli*, and *P. aeruginosa* [12,13].

The present study is predicated on the hypothesis that the formulation of nanoemulsion ethanol extract of basil leaves using a combination of Tween 80 and PEG 400 can produce an effective and stable topical preparation in inhibiting the growth of *Propionibacterium acnes.* To obtain the optimal formula, the Box-Behnken Design (BBD) experimental design method was used to evaluate the influence of variations in surfactant and co- surfactant concentrations on physical characteristic parameters, such as particle size, pH, and transmittance percentage [14].

The objective of this study is to develop and optimize the formulation of an ethanol extract of basil leaf nanoemulsion as an acne-fighting agent based on a nano-delivery system. This research is significant as a strategic step toward providing a safe, natural, and potentially antibiotic-resistance-reducing topical therapy alternative for acne treatment.

2 Experimental Procedures

2.1 Materials

The main material used was ethanol extract of basil leaves (*Ocimum basilicum* L.) obtained from PT. Lansida (Yogyakarta, Indonesia). Other materials included Virgin Coconut Oil (5 %) (Lansida®, Indonesia), Tween 80 (Matangi®, Indonesia), Polyethylene Glycol 400 (Petronas®, Malaysia), ascorbic acid (CSPC®, China) was included at a concentration of 0.05 % as an antioxidant to prevent oxidation of oil phytochemicals, benzoic acid (Lux Chemicals®, Indonesia) was added at a concentration of 0.1 % as a preservative to inhibit microbial growth during storage, and distilled water. For the antibacterial test, Mueller Hinton Agar (Merck®, Indonesia), 0.9 % physiological NaCl solution (Otsu®, Indonesia), dimethyl sulfoxide (DMSO) (Emsure®, Indonesia), and clindamycin discs were used as positive controls (Oxoid®, Indonesia).

Some of the main equipment included: analytical balance, UV-Vis spectrophotometer, FT-IR Spectrophotometer (Shimadzu®, Japan) pH meter (Ohaus® ST300, USA), Brookfield viscometer (Ametek®, USA), particle size analyzer (HORIBA SZ-100, Japan), magnetic stirrer, hotplate (Daihan® MSH-20D, South Korea), Ultra-Turrax homogenizer (Ika®, Jerman) centrifuge (Gemmy PLC®, Taiwan), and standard laboratory glassware.

2.2 Experimental

2.2.1 Extraction of Basil Leaf (*Ocimum basilicum* L.)

Fresh basil leaves were sorted, washed under running water, and oven-dried at 40 °C for 12 hours. The dried leaves were ground using a disk mill and sieved through a 60-mesh filter. The resulting powder was extracted with 96 % ethanol using an Ultra-Turrax homogenizer (1,000 rpm, 2 h) followed by maceration for 48 h at room temperature. The extract was filtered under vacuum, and the residue was re-extracted using the same procedure. The combined filtrates were concentrated with a rotary evaporator at 40 °C, followed by evaporation on a water bath to remove residual solvent. The dried extract was weighed to determine yield and stored at 4 °C in an airtight container until further use.

2.2.2 Characterization of Basil Leaf Extract

The basil leaf extract was characterized using Fourier-transform infrared spectroscopy (FTIR, Shimadzu®, Japan) in the wavenumber range of 4000–400 cm^{-1}. The sample was placed in a clean sample holder, and spectra were analyzed to identify characteristic functional groups corresponding to bioactive constituents.

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2.2.4 Antibacterial Activity Of Basil Leaf Extract

The antibacterial activity was tested against *Propionibacterium acnes* using the disc diffusion method. The basil leaf extract was dissolved in dimethyl sulfoxide (DMSO), which was selected as a universal solvent due to its lack of intrinsic antimicrobial activity and its ability to dissolve both polar and non-polar phytochemical constituents. Mueller–Hinton Agar (MHA) supplemented with 5 % defibrinated sheep blood was inoculated with *P. acnes* using a sterile cotton swab. Sterile paper discs (6 mm diameter) were impregnated with 50 µL of the extract solution at concentrations of 0.5 %, 1 %, 1.5 %, and 2 %. Commercial clindamycin 1 % discs (Oxoid®, Indonesia) and blank DMSO discs served as the positive and negative controls, respectively. The plates were incubated anaerobically at 37 °C for 48 hours. After incubation, the diameters of inhibition zones were measured in millimeters and expressed as mean ± standard deviation ($n = 3$) [15].

2.2.5 Determination of upper and lower concentration limits

This stage was performed to determine suitable concentration ranges of Tween 80 and PEG 400, functioning as the surfactant and co-surfactant system for subsequent nanoemulsion formulation. The upper and lower limits of the Tween 80 and PEG 400 concentrations were determined based on previous research. The Tween 80 concentration range (35 %-45 %) and the PEG 400 concentration range (15 %-25 %) [16,17]. These concentrations can produce stable nanoemulsions with small particle sizes (10–200 nm) and a low polydispersity index (<0.5).

2.2.6 Preparation of Basil Leaf Extract Nanoemulsion

The nanoemulsion was prepared using a modified spontaneous emulsification method, adapted from Mardiyanto [18]. The concentration of basil leaf extract in the oil phase was 1 % w/w, selected based on the results of the antibacterial activity test and the clarity observed during preformulation screening. The oil phase consisted of basil leaf extract dissolved in virgin coconut oil (VCO), to which Tween 80 was added and stirred using a magnetic stirrer at 500 rpm and a temperature of approximately 50 °C for 30 minutes. The aqueous phase was prepared by dissolving ascorbic acid and benzoic acid in distilled water, followed by the addition of PEG 400, and stirred under the same conditions. Subsequently, the oil phase was slowly introduced into the aqueous phase under continuous stirring at 1000 rpm for 60 minutes using a magnetic stirrer while maintaining the temperature at approximately 50 °C to facilitate the formation of a stable nanoemulsion.

2.2.7 Optimization of the Basil Leaf Extract Nanoemulsion

We analyzed the effect of the percentage concentration of surfactants and cosurfactants. Using Box-Behnken Design (BBD), we explored the relationship between two optimized factors (Tween 80 and PEG 400) and the response variables (The transmittance test (90 %-100 %) and the pH test (4.5-6.5). Response variable testing was conducted on 17 formulas, and the results were analyzed using Design-Expert software (version 13, Stat-Ease Inc., USA). The optimum formula was determined by processing the data from the Testing of the physical properties of the nanoemulsion preparations obtained from the experiment was based on The Design Expert recommended the high desirability value using the BBD method.

2.2.8 pH measurement

pH testing is performed using a pH meter. The meter is first calibrated using standard solutions with pH levels of 7, 4, and pH 10. After calibration, the electrode is immersed in the sample. A measurement result appears in the form of a number. pH testing for topical preparations must The pH range should be 4,5–6,5 which is consistent with skin pH [19].

2.2.9 Centrifugation Test

The centrifugation test was performed using a centrifuge. The nanoemulsion preparation was placed in a microtube containing 1.5 mL, then centrifuged for 30 minutes at a speed of 3000 rpm. The centrifugation test results are considered good and stable if there is no separation after centrifugation [20].

2.2.10 Viscosity Measurement

Viscosity measurements were performed using a Brookfield viscometer [21]. A total of 100 mL of sample was placed in a cup and installed on the available solvent trap. The Brookfield viscometer was set to 32 at a speed of 30 rpm with a spindle size of 4 for 30 seconds. The viscosity requirement for nanoemulsions is 10–2000 cPa.s.

2.2.11 Freeze-Thaw Test

This test was conducted by storing the nanoemulsion preparation for 24 hours at a cold temperature (4±2 °C), then transferring the preparation to a hot temperature (40±2 °C) for 24 hours (1 cycle). A total of 6 cycles were run for testing. The freeze-thawing stability requirements during storage were no change in color, odor, phase separation, and transmittance percentage [20].

2.2.12 Nanoemulsion Type Analysis

This test is conducted by mixing the nanoemulsion preparation with methylene blue on a watch glass, then stirring and observing visually. If the methylene blue dissolves in the formulation, this is an oil-in-water (O/W) nanoemulsion type, but if the formulation is a water-in-oil (W/O) type, the methylene blue does not dissolve or clumps on the surface of the nanoemulsion [22].

2.2.13 The Transmittance Test

The transmittance test was performed by dissolving 50 µL of nanoemulsion sample into 5 mL of distilled water until it was homogeneously mixed. The solution was then placed in a cuvette and measured using a UV-Vis spectrophotometer at a wavelength of 650 nm, with distilled water as the blank. This 1:100 dilution ratio refers to the method used by Helsawati [23]. Transmittance values in the range of 90–100 % indicate a clear solution and demonstrate the stability of the system.

2.2.14 Droplet size and distribution

The particle size and polydispersity index (PDI) were measured using a Horiba® SZ-100 particle size analyzer. One milliliter of nanoemulsion was diluted in 250 milliliters of Aqua Pro Injection and placed in a cleaned cuvette. The cuvette was placed in a sample holder for analysis. Measurements were performed in triplicate. Particle size values of ≤100 nm indicate a nanoemulsion, and a PDI of < 0.5 indicates homogeneous particle size distribution [24].

2.2.15 Droplet surface charge (Zeta Potential)

The zeta potential was measured using the same instrument (a Horiba SZ-100) at a temperature of 25 °C. One milliliter of the nanoemulsion formulation was diluted in 250 mL of Aqua Pro Injection and placed in a cuvette for analysis. The formulation is considered stable if its zeta potential value is ≥ ±30 mV [18].

2.2.16 Validation of the optimized Basil Leaf Extract Nanoemulsion

The optimum formula for basil leaf extract nanoemulsion was verified by reproducing it and testing it according to the response used. The experimental data was compared with the predicted results from the design expert, then the prediction error percentage was calculated to assess the accuracy of the optimization model. A prediction error percentage of <10 % is acceptable [25].

2.2.17 Skin Irritation Test

The skin irritation test was conducted with slight modifications according to the Guidelines for Dermal Irritation Test by OECD Test Guideline 404 [26].

Male Wistar rats weighing over 200 g were divided into two groups ($n = 3$ per group). Group I served as the control, consisting of a positive control (0.8 % formalin solution, v/v) and a negative control (nanoemulsion without active ingredients). Group II received the optimal basil leaf extract nanoemulsion as the test formulation.

Approximately 24 hours before application, a small area on the dorsal region of each rat was shaved without damaging the skin. A fixed amount of the test sample (0.5 g) was applied evenly to the exposed area and covered with sterile gauze for 4 hours. After removing the gauze, the application site was gently cleaned with distilled water.

Signs of irritation such as redness (erythema) and swelling (edema) were observed at 24 and 48 hours after application. The degree of irritation was evaluated according to the Draize scoring system, as recommended by OECD Test Guideline 404 [26]. The scoring criteria for erythema and edema are summarized in **Table 1.**

The Primary Irritation Index (PII) was then calculated using the following equation:

$$\text{PII} = \frac{\sum(\text{Erythema Score} + \text{Edema Score})}{\text{Number of observations}}$$

The PII values were interpreted as follows: 0.0–0.4 = non-irritant; 0.5–2.0 = mild irritation; ≥2.1 = moderate to severe irritation[26].

The purpose of this test is to determine whether the nanoemulsi formulation causes irritation when applied topically to healthy skin. This study has been approved and complies with the ethical guidelines for the use of laboratory animals, as evidenced by the ethical approval number issued by the Research Ethics Committee 0303/HRECC.FODM/II/2025.

Table 1 Standards for skin irritation study [25)]

Erythema and Eschar Formation	
Type	**Score**
No Erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation(injuries in depth)	4
Edema Formation	
Type	**Score**
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4

2.2.18 Antibacterial Activity of Basil Leaf Extract Nanoemulsion

The nanoemulsion used for antibacterial testing contained 1 % (w/w) basil leaf extract dissolved in an oil phase consisting of 5 % (w/w) VCO. The antibacterial activity of the optimum basil leaf extract nanoemulsion was assessed against *Propionibacterium acnes* using the agar disc diffusion method on MHA supplemented with 5 % sheep blood. Sterile paper discs (6 mm) were loaded with 50 µL of each sample (optimum nanoemulsion, clindamycin 2 µg/disc as positive control, and nanoemulsion base as negative control) and incubated anaerobically at 37 °C for 48 h. The inhibition zones were measured in millimeters (mean ± SD, $n = 3$).

2.3 Analysis method

All tests were conducted three times (n = 3), and the results are presented as Mean ± Standard Deviation (SD). Statistical analysis was performed using IBM SPSS Statistics version 29 and Microsoft Excel 2010 software. Data normality was tested using the Shapiro-Wilk test. Since the data were normally distributed, parametric tests were used: the paired t-test for within-group analysis and One-Way ANOVA for between-group analysis. A p-value ≤ 0.05 was considered statistically significant. Analysis of variance was also performed using Design-Expert® software version 13.

3 Results

3.1 Extraction of Basil Leaf (*Ocimum basilicum* L.)

The extraction of basil leaves (*Ocimum basilicum* L.) using 96 % ethanol through a combination of Ultra-Turrax homogenization and maceration produced a thick, dark green viscous extract with a characteristic aromatic odor and a bitter taste. From 1,042 g of dried basil leaf powder, 169.20 g of extract was obtained, corresponding to an extraction yield of 16.23 % (w/w). The extract was easily soluble in ethanol and DMSO, indicating the predominance of moderately polar compounds.

3.2 Characterization of Basil Leaf Extract

The FTIR spectrum of the basil leaf ethanol extract confirmed the presence of characteristic functional groups corresponding to bioactive secondary metabolites. The spectrum showed a broad absorption band at 3379.66 cm^{-1}, corresponding to hydroxyl (–OH) stretching vibrations, indicative of phenolic compounds. A peak at 1649.25 cm^{-1} was observed, corresponding to C=C stretching, which suggests the presence of aromatic ring structures. Additionally, the absorption at 1692.06 cm^{-1} indicated carbonyl (C=O) functional groups, reflecting the presence of flavonoids, terpenoids, and other bioactive constituents [10].

3.3 Antibacterial Activity of Ethanolic Basil Leaf Extract

The antibacterial activity of basil leaf extract against *Propionibacterium acnes* was evaluated prior to nanoemulsion formulation to determine the optimal extract concentration. The test employed the agar disk diffusion method using Whatman® No. 42 paper disc (6 mm diameter), which served as reservoirs for the extract. This method was selected due to its simplicity, cost-efficiency, and ability to provide measurable inhibition zones around the disks, particularly suitable for liquid samples [27]. Mueller-Hinton Agar (MHA) supplemented with fresh sheep blood was used as the culture medium, in line with WHO recommendations for anaerobic bacteria. The blood addition provides essential nutrients and mimics the biological environment of P. acnes, which is known to attract leukocytes as chemoattractants [28].

Results showed inhibition zones 8.57 ± 0.40 mm at 0.5 % extract concentration, 10.17 ± 0.15 mm at 1 %, 10.67 ± 0.20 mm at 1.5 %, and 11.47 ± 0.35 mm at 2 % (**Table 2**). Based on CLSI [6] standards, all concentrations exhibited weak antibacterial activity. This activity is attributed to the presence of secondary metabolites in the extract, especially flavonoids, which act by disrupting bacterial cell wall permeability and interfering with intracellular components such as DNA, microsomes, and lysosomes [11].

3.4 Determination of upper and lower concentration limits

Preliminary screening indicated that formulations containing less than 35 % Tween 80 resulted in phase separation within 24 hours, while concentrations above 45 % produced excessively viscous systems that were unsuitable for topical application. Similarly, PEG 400 concentrations below 15 % led to turbidity and poor emulsification, whereas levels exceeding 25 % decreased clarity and increased droplet coalescence. Based on these findings, the optimal concentration ranges of Tween 80 (35 %–45 %) and PEG 400 (15 %–25 %) were selected for subsequent optimization using the Box–Behnken Design (BBD).

3.5 Preparation of Basil Leaf Extract Nanoemulsion

The basil leaf extract nanoemulsion, prepared using a modified spontaneous emulsification method, produced a clear, homogeneous, and physically stable dispersion without any visible signs of phase separation or sedimentation after 24 hours of observation. The formulation exhibited a dark brown coloration attributed to the natural pigments present in the basil extract and possessed a smooth, uniform texture suitable for topical application. A 1 % concentration of basil leaf extract yielded a stable nanoemulsion with no visible precipitation or instability during preliminary evaluations. These findings confirm the effectiveness of Tween 80 and PEG 400 in reducing interfacial tension and establishing a robust interfacial film, thereby ensuring uniform droplet distribution and stability within the aqueous phase.

Table 2 Antibacterial Activity of Ethanol Extract of Basil Leaves (*Ocimum basilicum* L.)

Treatment	Inhibitory Zone Diameter (mm)			Average ±SD	Category (CLSI [6])
	I	II	III		
Clindamycin (2 µg/disk)	26.20	27.60	29.80	27.87 ± 1.81	Strong
DMSO	6.00	6.00	6.00	6.00 ± 0.00	No activity
0.5 %	8.20	9.00	8.50	8.57 ± 0.40	Weak
1 %	10.20	10.00	10.30	10.17 ± 0.15	Weak
1.5 %	10.50	10.90	10.60	10.67 ± 0.20	Weak
2 %	11.80	11.10	11.50	11.47 ± 0.35	Weak

Clindamycin (2 µg/disk) was used as the positive

Table 3 Results of Nanoemulsion Formula Optimization Design with Design Expert 13

Run[1]	Basil Leaf Extract (%)	Factor 1: Tween 80 (%)	Factor 2: PEG 400 (%)	Response 1: pH	Response 2: Transmittance (%)
NE 1	1	35	15	5.08	91.2
NE 2	1	40	20	5.70	96.2
NE 3	1	40	15	5.46	93.5
NE 4	1	35	20	5.49	94.9
NE 5	1	40	25	5.95	93.1
NE 6	1	40	25	5.95	94.6
NE 7	1	35	25	5.67	96.3
NE 8	1	40	20	5.70	95.8
NE 9	1	45	15	5.60	97.3
NE 10	1	40	20	5.70	94.5
NE 11	1	45	20	5.77	96.1
NE 12	1	45	25	5.88	97.5
NE 13	1	40	20	5.70	97.2
NE 14	1	45	20	5.77	95.4
NE 15	1	35	20	5.49	96.4
NE 16	1	40	15	5.46	96.9
NE 17	1	40	20	5.79	94.1

[1]*code name of combination of Tween 80 and PEG 400*

3.6 Optimization of the Basil Leaf Extract Nanoemulsion

Response Surface Methodology (RSM) using the Box-Behnken Design (BBD) was adopted to optimize the formulation and physical stability of basil leaf extract nanoemulsion. Tween 80 and PEG 400 were selected as the surfactant and co-surfactant, respectively. Tween 80, a high-HLB non-ionic surfactant (HLB 15), is ideal for forming oil-in-water (O/W) nanoemulsions, while PEG 400 was chosen for its hydrophilic nature and mid-chain hydrocarbon structure that enhances the flexibility of the interfacial film [29].

The concentrations of Tween 80 (X_1: 35–45 %) and PEG 400 (X_2: 15–25 %) were set as independent variables and entered into Design Expert software version 13 for optimization using the BBD model. The responses measured were pH and percent transmittance. A total of 17 experimental runs were generated by the software, each representing a unique combination of the two variables and formulated as a nanoemulsion containing basil extract (**Table 3**).

The pH value was evaluated as an indicator of topical product safety, with the optimal range being 4.5–6.5 to maintain skin barrier function and avoid irritation [19]. Transmittance, measured as a proxy for clarity and particle homogeneity, reflects nanoemulsion stability; values near 100 % suggest small, well-distributed droplets [23].

Interaction plots generated by the software demonstrated that increasing Tween 80 concentration led to a rise in pH, particularly at higher PEG 400 levels. This suggests a synergistic effect between the two excipients in elevating the system's pH. On the other hand, transmittance values increased significantly with higher Tween 80 levels under low PEG 400 conditions. However, at higher PEG 400 concentrations, further Tween 80 addition slightly reduced transmittance, possibly due to surfactant & co-surfactant imbalance disrupting colloidal uniformity.

These trends were visualized through 2D contour and 3D response surface plots (**Fig. 1**). In the pH plot, a gradient from blue to red indicated increasing pH values with rising concentrations of both components. In contrast, transmittance plots showed a green-to-yellow transition, reflecting areas of high clarity associated with specific formulation ratios. The optimized formula, predicted by the Design Expert software based on the highest desirability score (1.0), was obtained at 41.09 % Tween 80 and 23.35 % PEG 400. This formulation produced a pH of 5.88 and a percent transmittance of 95.9 %, both within the desired range for stable and skin-compatible cosmetic nanoemulsion [30, 31]

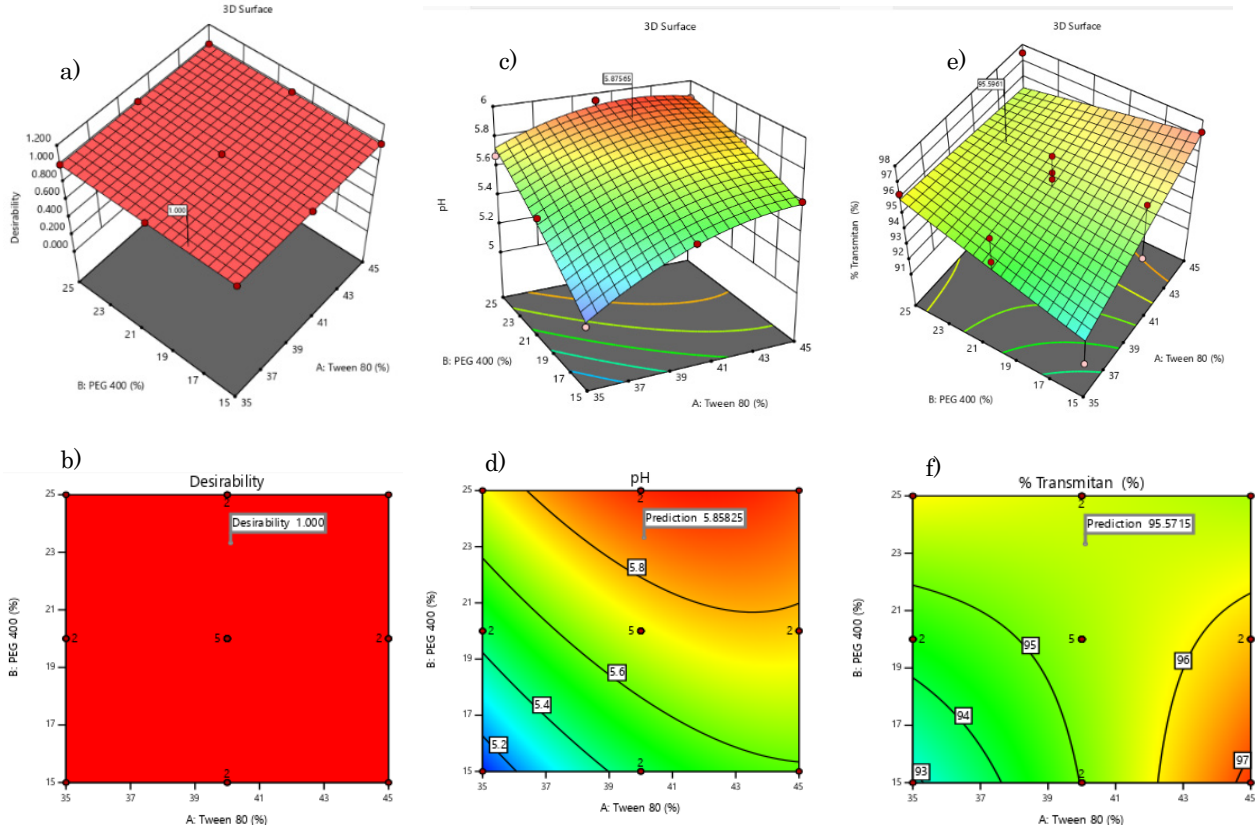


Fig. 1 Contour Plot Results of Tween 80 and PEG 400 on desirability (a,b) pH (c,d) and Percentage Transmittance (e,f)

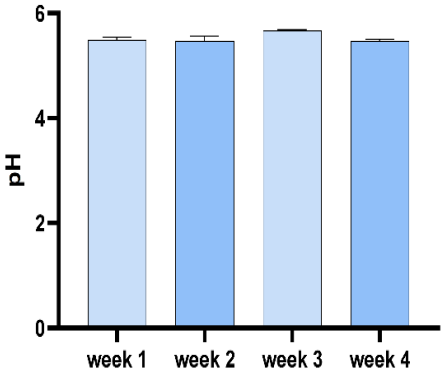


Fig. 2 pH test result of Basil Leaf Ethanol Extract Nanoemulsion

3.7 pH test

The pH measurement of basil (*Ocimum basilicum* L.) leaf extract nanoemulsion stored at room temperature for four weeks showed slight fluctuations. In the first week, the pH was recorded at 5.49 ± 0.05, followed by a slight decrease to 5.47 ± 0.09 in the second week. In the third week, a modest increase to 5.67 ± 0.02 was observed, before declining again to 5.47 ± 0.03 in the fourth week (**Fig. 2**). Despite these variations, all values remained within the acceptable pH range for topical formulations (4.5–6.5), indicating that the nanoemulsion remained safe and stable throughout the storage period 19. The storage period refers to four weeks at ambient temperature (25±2 °C).

Weekly intervals were chosen to simulate real-time storage stability over one month and to observe gradual physicochemical changes under normal conditions without inducing stress or accelerated degradation. Minor pH fluctuations were attributed to the redistribution of surfactant molecules, environmental temperature variations, and molecular rearrangement within the interfacial layer between oil and water phases. These slight changes did not indicate instability but reflected normal molecular adjustment during storage.

3.8 Centrifugation test

The centrifugation test of the basil leaf extract nanoemulsion showed no phase separation before or after testing, indicating excellent physical stability of the system. This method separates particles based on density under centrifugal force, where denser particles move downward while lighter ones move upward [32]. The test simulates the effects of gravity over a one-year period, and the absence of phase separation confirms that the nanoemulsion remains physically stable under accelerated stress conditions.

3.9 Viscosity measurement

In this study, the viscosity of basil leaf extract nanoemulsion showed fluctuations during four weeks of storage at room temperature. The viscosity increased from 700.00 ± 34.64 cPa·s in the first week to 846.66 ± 41.63 cPa·s in the second week, followed by a decrease to 780.00 ± 0.00 cPa·s in the third week and 646.66 ± 11.54 cPa·s in the fourth week (**Fig. 3**.).

Weekly intervals were selected to monitor gradual viscosity changes under real-time storage conditions, allowing evaluation of the nanoemulsion's physical stability throughout the one-month period. The observed fluctuations may be attributed to environmental temperature variations and molecular rearrangement within the interfacial film, which can affect droplet interactions and surfactant organization. The overall decreasing trend during storage could also result from partial droplet coalescence due to mild oil-phase crystallization or conformational relaxation of surfactant molecules [33]. These subtle variations, however, did not indicate instability, as the viscosity remained within the acceptable range for topical application.

3.10 Freeze-Thaw Test

Organoleptic evaluation during the freeze-thaw test indicated that physical attributes such as appearance, color, odor, and phase separation are critical indicators of formulation stability under extreme temperature fluctuations. The optimized nanoemulsion maintained its viscous liquid form, dark brown to blackish color, and characteristic herbal odor throughout six cycles, with no observed phase separation.

Transmittance measurements of the basil leaf extract nanoemulsion during six freeze-thaw cycles showed consistently high values: 97.9 ± 0.51, 97.4 ± 0.77, 96.4 ± 0.55, 96.4 ± 0.66, 96.3 ± 0.30, and 96.8 ± 0.81. The freeze-thaw test is a commonly used stress method to assess the physical stability of nanoemulsions, as drastic temperature shifts can induce phase separation, particle aggregation, or structural disruption, which in turn reduce clarity [34]. The stable and high transmittance values indicate that the nanoemulsion remained physically stable under thermal stress conditions.

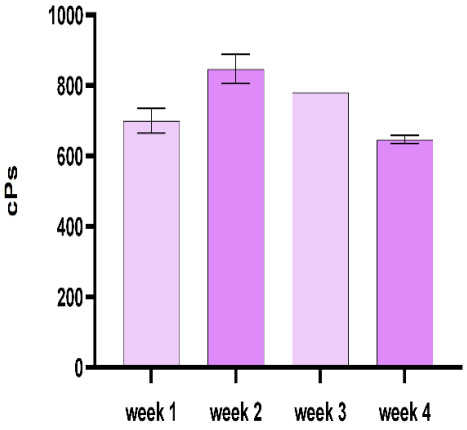


Fig. 3 Viscosity Graph of Basil Leaf Ethanol Extract Nanoemulsi

3.11 Nanoemulsion Type Analysis

The nanoemulsion type was preliminarily evaluated using a solubility test with methylene blue as an indicator. Methylene blue is a water-soluble dye commonly used to qualitatively indicate the nature of the continuous phase in emulsion systems. In the present study, methylene blue dissolved uniformly in the optimized formulation without precipitation, suggesting that the continuous phase was aqueous.

Based on this observation, the formulation was presumed to be an oil-in-water (O/W) nanoemulsion. However, it should be noted that solubility testing using lipophilic dyes such as Sudan III was not performed. Therefore, the determination of the nanoemulsion type in this study is limited to continuous phase indication and does not include detailed phase behavior or structural analysis. Oil-in-water nanoemulsions are currently preferred for drug delivery systems due to their ease of removal, water solubility, and safety [35].

3.12 The Transmittance Test

The transmittance values of the basil leaf extract nanoemulsion were relatively consistent across replicates, with values of 96.4 %, 97.7 %, and 96.5 %, yielding an average of 96.8 % ± 0.72. The high transmittance indicates that the particle size within the formulation is very small. Smaller particles allow light to pass through the system with minimal scattering, resulting in a clear appearance and higher transmittance values [16]. During measurement, distilled water was used as a blank due to its particle-free composition, which ensures unobstructed light transmission.

3.13 Droplet size and distribution

The average particle size of the basil leaf extract nanoemulsion was 13.80 ± 0.36 nm, which falls within the nanometric range of 10–200 nm. This small size enhances surface area, improves drug release, and increases bioavailability. Tween 80, as a surfactant, reduces interfacial tension by adsorbing at the oil–water interface and forming a stabilizing monolayer around the dispersed oil droplets, while PEG 400 acts as a co-surfactant that enhances the flexibility of the interfacial film, improves droplet packing, and prevents phase separation, thereby contributing to the overall stability of the nanoemulsion system [36].

The measured polydispersity index (PDI) was 0.18 ± 0.04, indicating a narrow and uniform particle size distribution. A PDI below 0.5 reflects a monodisperse system, whereas values above 0.5 indicate heterogeneity [32]. The low PDI confirms the formation of a stable and homogeneous nanoemulsion, which reduces the risk of physical instability such as creaming and cracking [37].

3.14 Droplet surface charge (Zeta Potential)

Zeta potential reflects the overall surface charge of dispersed particles and is a key predictor of nanodispersion stability. High absolute zeta potential values (positive or negative) promote electrostatic repulsion, preventing particle aggregation. In contrast, low zeta potential increases the likelihood of flocculation or sedimentation [38]. In this study, the average zeta potential of the basil leaf extract nanoemulsion was – 20.00 ± 0.80 mV. A zeta potential of ±30 mV generally indicates good long-term stability, while values around ±20 mV suggest moderate or short-term stability. Values below ±5 mV typically lead to rapid aggregation [39].

Zeta potential is influenced by several factors, including pH, ionic strength, temperature, and the presence of ionic surfactants or polymers. pH is particularly critical: low pH tends to produce positive zeta potential, while high pH leads to lower or negative values. In this formulation, the use of nonionic surfactant Tween 80, which lacks electrical charge, contributed to the relatively low zeta potential [24]. Nevertheless, the nanoemulsion demonstrated good physical stability, as evidenced by the absence of phase separation during centrifugation testing.

Table 4 Validation of the optimized Basil Leaf Extract Nanoemulsion

Response Variable	Predicted Value	Formula Evaluation			Mean±SD	% Prediction Error
		Rep (1)	Rep (2)	Rep (3)		
pH test	5.87	5.49	5.44	5.54	5.49 ± 0.05	6.92
Transmittance percentage	95.6	96.4	97.7	96.5	96.8 ± 0.72	1.23

Note: the results were presented as mean ±SD (n='3)

Table 5 Skin Irritation Test Results of Basil Leaf Extract Nanoemulsion

Treatment	Erythema Score (0 h / 24 h / 48 h)	Edema Score (0 h / 24 h / 48 h)	Primary Irritation Index (PII)	Category (OECD 404) [30]
The nanoemulsion base without basil extract	0 / 1 / 0	0 / 0 / 0	**1/6 = 0.16**	Non-irritant
Formalin 0.8 %	0 / 2 / 2	0 / 1 / 1	**6/6 = 1.00**	Mild–moderate irritation
Test sample	0 / 1 / 1	0 / 0 / 0	**2/6 = 0.33**	Non-irritant

The nanoemulsion base without basil extract was used as the negative control, formalin (0.8 %) served as the positive control, and the test sample was the basil leaf extract nanoemulsion containing 1 % extract.

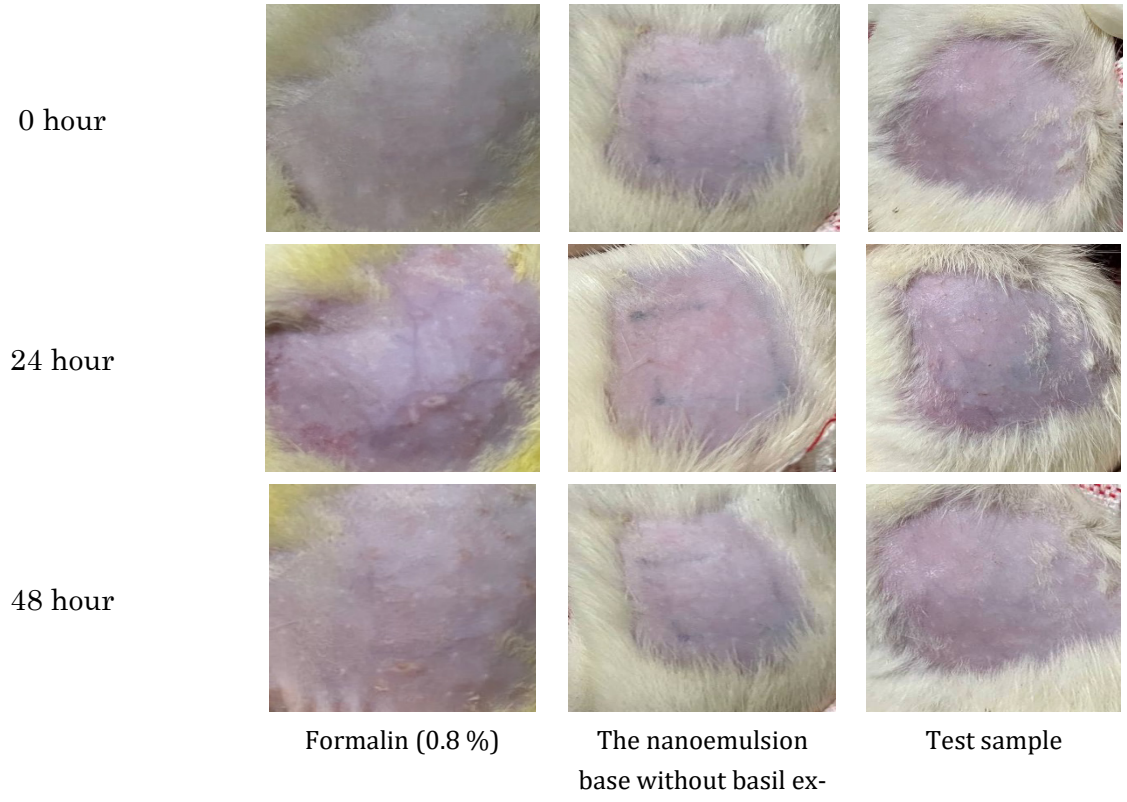


Fig.4 Comparison of mean Primary Irritation Index (PII) values between the nanoemulsion base without basil extract was used as the negative control, formalin (0.8 %) served as the positive control, and the test sample was the basil leaf extract nanoemulsion containing 1 % extract.

3.15 Validation of the optimized Basil Leaf Extract Nanoemulsion

To verify the reliability of the predicted optimum formula, experimental validation was conducted and compared with the predicted values. The percentage prediction error analysis showed

that the pH response had an error of 6.92 %, while the transmittance response showed an error of 1.23 % (**Table 4**). These values indicate that the experimental results were in close agreement with those predicted by the Design Expert model, confirming the model's accuracy. Prediction errors below 10 % are generally acceptable for validating predictive models in formulation studies [25].

Table 6. Antibacterial Activity of Basil Leaf Extract Nanoemulsion

Treatment	Inhibitory Zone Diameter (mm)			Average ±SD	Category (CLSI) [6]
	I	II	III		
Positive Control	27.30	26.90	26.00	26.73 ± 0.66	Strong
Negative Control	9.50	10.20	9.70	9.80 ± 0.36	Weak

Clindamycin (2 µg/disk) was used as the positive control, while the nanoemulsion base without basil extract served as the negative control. The test sample was the basil leaf extract nanoemulsion containing 1 % extract.

3.16 Skin Irritation Test

The results of the skin irritation test are presented in **Table 5** and **Fig. 4.** The basil leaf extract nanoemulsion showed no visible signs of erythema or edema during 48 hours of observation. The mean Primary Irritation Index (PII) value of the nanoemulsion was 0.33 which was comparable to the negative control (PII = 0,16) and significantly lower than the positive control (0.8 % formalin solution, PII = 1,00). According to OECD 404 guidelines [26], formulations with PII ≤ 1,0 are classified as non-irritant. Therefore, the nanoemulsion formulation was categorized as non-irritating and considered safe for topical application. The negative control also showed no reaction, confirming that the excipients were safe, while the positive control (0.8 % formalin) produced moderate erythema and edema, validating the sensitivity of the test.

The low irritation index of the nanoemulsion can be attributed to its biocompatible components, such as Tween 80 (a mild non-ionic surfactant), PEG 400 (a co-surfactant with good skin tolerance), and Virgin Coconut Oil (VCO), which also acts as an emollient. The nanoemulsion structure enhances dispersion of active ingredients and reduces the concentration of extract directly contacting the skin surface, thereby minimizing the risk of irritation.

3.17 Antibacterial Activity of Basil Leaf Extract Nanoemulsion

The antibacterial activity test using the disk diffusion method showed that the basil leaf extract nanoemulsion (1 % extract) produced an inhibition zone of 12.30 ± 0.28 mm, classified as weak antibacterial activity. In comparison, the positive control (clindamycin 2 µg/disk) exhibited a significantly larger inhibition zone of 26.73 ± 0.66 mm (strong activity), while the negative control (nanoemulsion base without extract) showed a zone of 9.80 ± 0.36 mm, also categorized as weak (**Table 6**).

Clindamycin was selected as the positive control due to its proven effectiveness against Gram-positive and anaerobic bacteria, including *Propionibacterium acnes* [40]. The slight antibacterial activity observed in the negative control can be attributed to minor contributions from the nanoemulsion base, which contained 5 % virgin coconut oil (VCO) and 0.1 % benzoic acid. The antibacterial effects of VCO are caused by the presence of medium-chain fatty acids, namely lauric acid, capric acid, and caprylic acid, which can damage the cell walls of bacteria, especially Gram-positive strains [41]. However, these effects are strongly concentration-dependent. According to[42], VCO demonstrates significant antibacterial activity against *P. acnes* only when used at concentrations of 20–30 % in topical emulgel formulations. Given that the present formulation contained only 5 % VCO, its intrinsic antibacterial effect is considered negligible.

Although specific antibacterial testing of basil extract dissolved in VCO at the same concentration was not performed in this study, literature evidence indicates that VCO alone shows only weak antibacterial activity at such low levels. Consequently, the antibacterial activity observed in the basil leaf extract nanoemulsion primarily stems from the inherent properties of the basil extract itself, as indicated by the extract solution (**Table 2**). The larger inhibition zone observed for the nanoemulsion compared to the negative control reflects the contribution of the basil extract; however, the nanoemulsion did not demonstrate a significant enhancement of antibacterial activity when compared with the extract solution. The role of the nanoemulsion system in this study is therefore limited to improving dispersion homogeneity and physical stability of the formulation, rather than acting as an enhancer of antibacterial potency. The combination of VCO, surfactant (Tween 80), and co-surfactant (PEG 400) promotes uniform droplet formation, reduces interfacial tension, which supports the formation of a physically stable dispersion system suitable for topical antibacterial formulations.

Normality and homogeneity tests showed that the data were normally distributed and homogeneous (p-value > 0.05), allowing the use of parametric statistical analysis. One-way ANOVA revealed a significant difference ($p < 0.05$) among the tested groups, primarily associated with the presence of the basil leaf extract.

Previous studies have reported that nanoemulsions are commonly employed as delivery systems in topical formulations due to their favorable physicochemical properties[43]. However, their antibacterial effectiveness remains dependent on the concentration of active compounds and the susceptibility of the tested microorganism.

Although the inhibition zone of the basil leaf extract nanoemulsion did not differ significantly from that of the extract solution, the nanoemulsion system offers formulation-related advantages. The encapsulation of hydrophobic bioactive compounds within the oil phase (VCO) and surfactant supports uniform dispersion of the extract within the formulation. The nanometric droplet size (typically <200 nm) also enhances surface area contact, which is considered advantageous for the design of topical formulations targeting acne-related bacteria, where conventional extract solutions have limited accessibility. In addition, nanoemulsion systems may contribute to improved physical stability and protection of sensitive phytoconstituents during topical application. Therefore, even though the direct inhibition zone was comparable, the nanoemulsion provides a physically stable and suitable formulation platform for topical use.

4 Conclusion

The optimal formula for the nanoemulsion of basil leaf extract was obtained with a Tween 80 concentration of 41.09 % and PEG 400 concentration of 23.35 % based on the prediction of the Design Expert 13 software. The resulting nanoemulsion had an average particle size of 13.80 ± 0.36 nm with a PDI value of 0.18 ± 0.04, indicating a homogeneous particle size distribution. The zeta potential value of –20.00 ± 0.80 mV indicated adequate electrokinetic stability. The antibacterial activity test using the disk diffusion method yielded an inhibition zone diameter of 12.30 ± 0.28 mm, categorized as weak antibacterial activity.

Ethical approval

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Research Ethics committee (REC) of Faculty of Medicine, Abdurrab University, Riau, Indonesia (No:0303/HRECC.FODM/II/2025).

Credit T authorship contribution statement

Benni Iskandar: Conceptualization, methodology, investigation, data curation, writing and funding acquisition. Rusnita Putri: Writing-review & editing, methodology, investigation, data curation. Ching-Peng Wei: Methodology, writing-review & editing. Fitri Renovita: Conceptualization, methodology, writing-review & editing.

Declaration of Competing Interest

The authors state that they have no known competing financial or personal interests that could have impacted the work presented in this paper.

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