ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF NANOEMULSION FORMULATED FROM *Baeckea frutescens* AND *Citrus x microcarpa* ESSENTIAL OILS

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Abstract: The use of essential oils in the development of natural pharmaceutical and cosmetic products as antioxidants and antibacterial agents is currently gaining popularity. Research on combining *Baeckea frutescens* L. essential oil with key lime (*Citrus x microcarpa* Bunge) peel waste remains limited despite their individual antimicrobial efficacy and potential synergistic effects as natural preservatives. This study aims to evaluate the antioxidant and antibacterial activities of essential oils from sapu-sapu plants and key lime fruit waste combined in nanoemulsion preparations. The essential oils were obtained through steam distillation and formulated into two nanoemulsion preparations, F1 (EOB:EOC, 40:60) and F2 (EOB:EOC, 60:40). The characteristics of F1 showed a particle size of 7.06 nm with a PDI of 0.0067, while F2 had a size of 14.64 nm with a PDI of 0.1528, indicating that both formulations showed good stability and homogeneity. The antioxidant activity test results showed an IC_{50} of 3.3305 ppm for F1 and 4.5234 ppm for F2, indicating that F1 has stronger antioxidant activity through the DPPH method. The results of antibacterial activity tests against *Staphylococcus aureus, Staphylococcus epidermidis*, *Escherichia coli,* and *Cutibacterium acnes* showed that both formulas had moderate to strong activity against all test bacteria, with F1 providing a wider inhibition zone than F2. The essential oil of sapu-sapu and the essential oil derived from key lime fruit waste, when combined in a nanoemulsion formulation, demonstrate potential as natural antibacterial and antioxidant agents, offering promising candidates for sustainable bioactive ingredients in pharmaceutical and food applications.

Keywords: Nanoemulsion; essential oil; *Baeckea frutescens; Citrus x microcarpa*; antioxidant; antibacterial

Abstrak: Penggunaan minyak atsiri dalam pengembangan produk farmasi dan kosmetik alami sebagai agen antioksidan dan antibakteri saat ini semakin populer. Penelitian mengenai pengombinasian minyak atsiri *Baeckea frutescens* L. dengan minyak atsiri dari limbah kulit jeruk nipis (*Citrus x microcarpa* Bunge) masih terbatas, meskipun masing-masing telah diketahui memiliki efektivitas antimikroba serta potensi efek sinergis sebagai pengawet alami. Penelitian ini bertujuan untuk mengevaluasi aktivitas

antioksidan dan antibakteri dari minyak atsiri tanaman sapu-sapu dan limbah buah jeruk nipis yang dikombinasikan dalam sediaan nanoemulsi. Minyak atsiri diperoleh melalui metode destilasi uap dan diformulasikan menjadi dua sediaan nanoemulsi, yaitu F1 (EOB:EOC = 40:60) dan F2 (EOB:EOC = 60:40). Karakteristik F1 menunjukkan ukuran partikel sebesar 7.06 nm dengan nilai PDI 0.0067, sedangkan F2 memiliki ukuran partikel 14.64 nm dengan nilai PDI 0.1528, yang mengindikasikan bahwa kedua formulasi memiliki stabilitas dan homogenitas yang baik. Hasil uji aktivitas antioksidan menunjukkan nilai IC_{50} sebesar 3.3305 ppm untuk F1 dan 4.5234 ppm untuk F2, yang menandakan bahwa F1 memiliki aktivitas antioksidan yang lebih kuat berdasarkan metode DPPH. Hasil uji aktivitas antibakteri terhadap *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, dan *Cutibacterium acnes* menunjukkan bahwa kedua formula memiliki aktivitas sedang hingga kuat terhadap seluruh bakteri uji, dengan F1 menghasilkan zona hambat yang lebih luas dibandingkan F2. Minyak atsiri sapu-sapu dan minyak atsiri yang berasal dari limbah buah jeruk nipis, ketika dikombinasikan dalam formulasi nanoemulsi, menunjukkan potensi sebagai agen antibakteri dan antioksidan alami, sehingga berpeluang menjadi kandidat bahan bioaktif berkelanjutan untuk aplikasi farmasi dan pangan.

Kata kunci: Nanoemulsi; minyak essensial; *Baeckea frutescens; Citrus x microcarpa*; antioksidan; antibakterial

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Introduction

Skin and gastrointestinal problems are common health issues caused by pathogenic bacterial infections. Some pathogenic bacteria often associated with these problems are *Staphylococcus aureus, Staphylococcus epidermidis, Cutibacterium acnes,* and *Escherichia coli* (Ham *et al.*, 2023; Di Francesco. *et. al*., 2025). *S. aureus* and *S. epidermidis* are known to be the main causes of skin and wound infections due to their ability to form biofilms and increase resistance to antibiotics (Matthew, 2024). Skin infections can also be triggered by *C. acnes*, which plays a role in the pathogenesis of acne through the production of lipase that breaks down sebum into free fatty acids, causing inflammation and further bacterial growth (Barnard *et al.*, 2020).

Bacterial infections are also known to affect the digestive system and trigger serious health problems. *E. coli* is a pathogenic bacterium that can infect the digestive tract and is the main cause of severe diarrhea or gastroenteritis, especially in individuals with weak immune systems (Gambushe *et. al.*, 2022). Infections caused by *E. coli* are often difficult to treat due to high bacterial resistance to antibiotics, thus requiring effective alternative treatments based on natural ingredients to suppress resistance. In addition to bacterial infections, the human body is also susceptible to damage from free radicals. Free radicals are unstable molecules that can damage cells, accelerate aging, and increase the risk of

degenerative diseases such as cancer and cardiovascular disease. Agents or compounds with antioxidant activity are known to play a role in counteracting free radicals, thereby helping to maintain skin health and accelerate the healing of wounds caused by bacterial infections (Michalak, 2022).



Figure 1. *Baeckea frutescens* L.



Figure 2. *Citrus x microcarpa* Bunge.

Essential oil from the sapu-sapu plant (*Baeckea frutescens* L.) has been extensively studied for its various active compounds and is known to have antibacterial and antioxidant properties. *B. frutescens* essential oil contains flavonoids, phenols, and terpenoids that can inhibit the growth of pathogenic microorganisms and ward off free radicals (Huong *et al.*, 2023). Meanwhile, key lime peel waste has also been studied and found to be rich in essential oils that have not been optimally utilized, even though it is known to contain terpenoids such as limonene, which has antimicrobial activity because it can inhibit the synthesis of nucleic acids and bacterial cell walls (Quoc & Phuong, 2025). Research by Septiani *et al.*, (2024) revealed that orange peel essential oil from Bengkulu has strong antioxidant activity with an IC_{50} value of 28.92 ppm, indicating its ability to inhibit oxidation. Essential oil from key lime peel from Pontianak has also been proven to have antibacterial activity against *S. aureus* (9-11 mm) and *Staphylococcus mutans* (7-10 mm), thus potentially inhibiting acne growth (Ropiqa *et. al.*, 2023). According to Roanisca *et al.*, (2021), key lime (*Citrus x microcarpa* Bunge) is an endemic plant of Bangka Belitung that not only provides a sour taste but also has medicinal properties for coughs.

Essential oils are oils extracted through distillation from natural materials (Pipatpaiboon *et al.*, 2025). Given the limitations of conventional essential oils, nanotechnology offers a promising strategy to improve stability and bioavailability. Therefore, to improve solubility, stability, bioavailability and controlled release to the appropriate physiological target, a coating technique is required (Santo *et al.*, 2021).

Nanoemulsion is a coating technique for active compounds (liquid, solid, or gas) using polymers made on a nanometer scale, ranging from 1 to 1000 nm. Nanotechnology can increase the solubility of drug compounds and improve their pharmacokinetic profile, thereby enabling dose reduction (Liu, *et. al.*, 2024). In addition, the size of nanoparticles can support the stability of drug compounds

during transport and distribution, thereby optimizing the pharmacological effects of active compounds.

The application of nanotechnology has been proven to increase bioavailability, stability, protect active compounds from degradation, improve skin penetration and enable controlled release, thereby enhancing the antibacterial and antioxidant effectiveness of a compound (Safta *et. al.*, 2024). However, limited studies have explored the synergistic effect of combining *B. frutescens* and *C. microcarpa* essential oils in nanoemulsion form to enhance their biological activities. Therefore, this study aims to develop nanoemulsion formulations from a combination of *Baeckea frutescens* L. and *Citrus* × *microcarpa* Bunge essential oils as an innovation in the development of natural-based antibacterial and antioxidant agents. The use of plant-based essential oils supports both halal product development and sustainable resource utilization, contributing to the advancement of eco-friendly bioactive formulations.

Methods

Materials

The sample used in this study was sapu-sapu leaves (*Baeckea frutescens* L.) which was obtained from Heath Forest Area of the East Coast of Bangka Belitung, Indonesia, and key lime fruit waste (*Citrus x microcarpa* Bunge.) which was obtained from Mirando Orange Syrup production house in Gerunggang District, Pangkalpinang City, Bangka Belitung, Indonesia. Bacteria used in the antibacterial activity test was isolated from *E. coli, S. epidermidis, S. aureus* and *C. acnes* (collection of the Microbiology Laboratory of the Biology Department of Bangka Belitung University). In addition, this study used nutrient agar (NA), virgin coconut oil (VCO), propylene glycol, Tween 80, blank antimicrobial susceptibility disks (Oxoid, New York), barium chloride ($BaCl_2$), sulfuric acid (H_2SO_4), ethanol, methanol, DPPH (2,2-Diphenyl-1-picrylhydrazyl) (HiMedia, India), ascorbic acid, physiological sodium chloride (NaCl), and distilled water (Onemed, America). All chemicals used were of analytical grade (Merck, Germany), unless otherwise specified.

Instrumentations

Key instruments included a UV-Vis spectrophotometer (Shimadzu, Japan), particle size analyzer (Biobase, China), and homogenizer (IKA, Germany). Additional equipment comprised a pH meter (Hanna, USA), incubator (Heraeus, Germany), laminar airflow cabinet (Esco Scientific, Singapore), and micropipettes of varying volumes (Biorad and Watson, USA).

Extraction of Essential Oil from Sapu-sapu Leaf Samples and Key Lime Fruit Waste

This study employed a laboratory experimental method with descriptive analysis. Essential oil extraction was carried out using steam distillation with a 36 cm diameter Zebra Thailand distillation apparatus made of SUS 304 stainless steel.

Samples of sapu-sapu leaves or key lime fruit waste (5 kg) were placed on a sieve in a kettle containing 2 L of water without direct contact, and the process was performed in duplicate. The heating process was conducted at a steam temperature of 100–110°C and a flame temperature of 150–300°C for 4 hours until the distillate was obtained. The essential oil and hydrosol were collected using a separating funnel, then separated, and the essential oil yield was calculated (Elicia, *et.al.*, 2024; Tafdila, 2025). The yield of essential oil was calculated as shown in Equation 1:

$$\text{Yield (\%)} = \frac{\text{Mass of Essential Oil (g)}}{\text{Mass of Simplisia (g)}} \text{ x } 100\% \quad \text{..... (1)}$$

Nanoemulsion Formulation of Combination of Baeckea frutescens and Citrus x microcarpa Essential Oils

Nanoemulsion formulation was conducted using High-Pressure Homogenization (HPH), a method known for producing uniform droplet sizes and stable formulations with better reproducibility than conventional emulsification techniques. The oil phase consisting of essential oils was dispersed in surfactants with the addition of *Virgin Coconut Oil* (VCO) and cosurfactants, then homogenized using a homogenizer at a speed of up to 5000 rpm assisted by sonication to reduce the particle size for 30 minutes. The aqueous phase in the form of distilled water was added to the mixture. Characterization of the nanoemulsion preparation included organoleptic evaluation, homogeneity, pH measurement using a pH meter, spreadability test, viscosity measurement using a Brookfield viscometer and particle size analysis using a *Particle Size Analyzer* (PSA) (Ardila, 2024).

Table 1. Nanoemulsion Formulation

Materials	Function	F1 (g)	F2 (g)
Essential Oils of *Baeckea frutescens* (EOB) and *Citrus x microcarpa* Bunge (EOC)	Active Substance	(40:60) 1	(60:40) 1
Virgin Coconut Oil (VCO)	Emollient	1	1
Tween 80	Surfactant	18	18
Propylene Glycol	Cosurfactant	5	5
Distilled Water	Solvent	25	24

Determination of Particle Size and Polydispersity Index (PDI) Value

Particle size measurement was performed using the dynamic light scattering (DLS) method with a *Particle Size Analyzer* (PSA). A total of 10 mg of sample was dissolved in distilled water and sonicated for 10 minutes to obtain a homogeneous solution. The sample solution was then placed in a cuvette and inserted into the PSA holder (Hidayat, 2025).

Antibacterial Activity Test

Biosafety and Ethical Considerations

This research employed biosafety level 2 practices when working with pathogenic bacterial strains to mitigate occupational exposure hazards. Personnel engaged in microbiological work wore complete protective gear including laboratory coats, latex gloves, and face protection throughout experimental activities. Contaminated materials and spent bacterial cultures were subjected to autoclave treatment at 121°C for 20 minutes to ensure complete sterilization before waste removal, following the institution's established biosafety procedures.

Tool Sterilization

All equipment used is washed, dried, and sterilized to eliminate any form of microorganism contamination that could affect the validity of the research results. Metal equipment such as tweezers, forceps, and spatulas are sterilized using the flaming method over a spirit flame until they reach incandescent conditions. Plastic equipment is sterilized using 70% ethanol (v/v). Meanwhile, glass equipment is sterilized using an autoclave at a temperature of 121 °C with a pressure of 2 atm for 20 minutes. Before the autoclave sterilization process, each tool is wrapped in paper, aluminum foil, or heat-resistant plastic to maintain sterile conditions after sterilization. The entire procedure is carried out aseptically in a laminar air flow that has been cleaned with 70% alcohol and irradiated with UV light for 30 minutes.

Nutrient Agar (NA) Media Preparation

Nutrient agar (NA) medium is used as a substrate for rejuvenating test bacteria, prepared by dissolving 2.4 grams of nutrient broth and 4.5 grams of agar in 300 mL of sterile distilled water in an Erlenmeyer flask. The medium is heated using a hot plate with a magnetic stirrer until the solution boils and appears clear, indicating complete solubility. Prepare slanted agar by pouring 6 mL of NA medium into a sterile test tube. All containers containing the medium are covered with cotton plugs lined with gauze and aluminum foil. Media sterilization was performed using an autoclave at 121°C for 20 minutes. For slant agar, the test tubes were positioned at an angle of 30-45° during the solidification process.

Rejuvenation of Test Bacteria

Isolates of *Cutibacterium acnes, Staphylococcus epidermidis, Staphylococcus aureus,* and *Escherichia coli* bacteria were inoculated onto slanted agar media using the zigzag streak plate technique. One bacterial loop was transferred to the agar surface, followed by incubation under anaerobic conditions for 24 hours at 37°C (Otite, *et.al.*, , 2024).

Test Bacterial Suspension

The suspension was prepared using bacterial colonies from a 24-hour culture suspended in 9 mL of sterile 0.9% (w/v) sodium chloride solution (LaPierre *et al.*, 2020). The turbidity of the bacterial suspension was standardized using the

McFarland method. The bacterial suspension and McFarland 0.5 standard were placed in screw-cap tubes with identical dimensions and volumes to facilitate accurate turbidity comparisons. Turbidity evaluation was performed by placing both tubes on a Wickerham card (a sheet with a white background and black lines). Prior to comparison, both the bacterial suspension and the McFarland standard were homogenized using a vortex mixer to ensure even cell distribution. This process was carried out to obtain a bacterial concentration equivalent to 1.5 x 10% CFU/mL, which is the cell density represented by the McFarland 0.5 standard.

Preparation of Test and Control Solutions

A 100% (w/v) solution was prepared by pipetting 1 mL of essential oil. A clindamycin positive control solution was prepared by mixing 250 mg of clindamycin into 5 mL of distilled water, then taking 1 mL and storing it in a positive control Petri dish.

Antibacterial Activity Testing

Antibacterial activity was evaluated using the Kirby-Bauer disc diffusion method. Antibacterial test media were prepared using the pour plate technique, in which 1 mL of bacterial suspension and ± 20 mL of NA media were inoculated into a sterile 15 cm x 2.5 cm Petri dish, then homogenized and allowed to solidify. Then, sterile 6 mm diameter discs were soaked in each concentration of test solution and control solution for 15 minutes to ensure optimal absorption. The discs were then placed on the surface of the inoculated medium using sterile tweezers, applying light pressure to ensure good contact. The distance between the discs was 3 cm, while the distance from the edge of the dish was 2 cm. The test was replicated twice. The test media was tightly covered using wrapping wrap to create anaerobic conditions. After 18 hours of incubation at 37°C, the diameter of the inhibition zone in the form of a clear zone formed around the paper discs was measured using calipers and calculated using the formula. The antibacterial assay was performed in triplicate (Mehmood, *et.al.*, 2024).

$$\text{Inhibition Zone (mm)} = \frac{(\mathbf{a}-\mathbf{c})+(\mathbf{b}-\mathbf{c})}{\mathbf{2}} \quad \text{.....(2)}$$

Description:
a = vertical diameter,
b = horizontal diameter,
c = diameter of the paper disc.

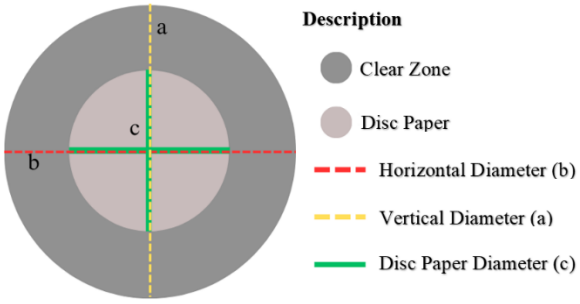


Figure 3. The measurement of the inhibition zone is performed (Hidayat, 2025)

In addition, the antibacterial effectiveness of the nanoemulsion prepared from *Baeckea frutescens* and *Citrus x microcarpa* Bunge essential oils was determined by applying the equation to compare its activity with that of the antibiotics used as references (Hidayat, 2025).

$$E = \frac{D}{Da} x\ 100\% \quad \text{.....(3)}$$

E = Antibacterial effectiveness (%)
D = Diameter of inhibition zone of nanoemulsion (mm)
Da = Diameter of antibiotic inhibition zone (mm)

Table 2. Antibacterial Inhibition Zone Strength Level (Emelda *et al*., 2021)

Antibacterial Intensity	**Zone of Inhibition (mm)**
Very Strong	> 20 mm
Strong	10 - 20 mm
Currently	5 – 10 mm
Weak	< 5mm

Antioxidant Activity Test

The IC_{50} value in the DPPH radical scavenging assay was determined using UV–Vis spectrophotometry based on a method adapted from Baliyan *et. al.* (2022), in which a series of sample dilutions were mixed with the DPPH solution, allowed to stand in the dark for a set incubation time, and then measured at 517 nm.

Making Test Solutions

A 1000 ppm essential oil stock solution was prepared by dissolving 1 mL of essential oil in p.a. methanol in a 10 mL volumetric flask. From this stock solution, concentrations of 10, 15, 20, 25, and 30 ppm were prepared by pipetting 1 mL and diluting to the mark with p.a. methanol.

Preparation of DPPH Solution

Weigh 2.5 mg of DPPH, then dissolve it in p.a. methanol to a volume of 25 mL. Store this solution in a container covered with aluminum foil.

Preparation of Blank Solution

A total of 2 mL of DPPH solution was pipetted into a test tube, 2 mL of methanol was added, then vortexed for 30 seconds and incubated at 37º for 30 minutes. The absorbance of this solution was measured at a wavelength of 517 nm using a UV-Vis Spectrophotometer.

Determination of IC_{50} Value

Each test solution was pipetted into a test tube in a volume of 1 mL, then 1 mL of DPPH solution and 2 mL of p.a. methanol were added, followed by vortexing for 30 seconds. After homogenization, the solution was incubated at 37ºC for 30 minutes and the absorbance was measured at a wavelength of 517 nm. The IC_{50} value was calculated using linear regression from the curve showing the relationship between the percentage of inhibition and the concentration of the test solution. Testing was performed using a UV-Vis spectrophotometer to measure the absorbance of the DPPH solution after adding the sample. DPPH Inhibition Percentage:

$$\text{Inhibition (\%)} = \frac{(Ak-As)}{Ak} \text{x } 100\% \quad \text{.....(4)}$$

Description:
Ak = Absorbance of DPPH solution without sample
As = Absorbance of DPPH solution with sample

Next, calculate the IC_{50} value by entering the inhibition percentage of each concentration using a linear regression equation.

$$y = a + bx \quad \text{.....(5)}$$

Description:
y = % inhibition, i.e., 50
x = IC_{50} value

Table 3. Antioxidant Strength Levels Using the DPPH Method (Tafdila, 2025)

Antioxidant Intensity	**IC_{50} Value**
Very Strong	< 50 µg/mL
Strong	50 - 100 µg/mL
Currently	100 - 250 µg/mL
Weak	250 - 500 µg/mL

Result and Discussion

Nanoemulsion Formulation

Nanoemulsion formulation is a stage in combining two immiscible phases, namely the oil phase and the water phase, with the help of surfactants to form a stable nano-sized colloidal system (<200 nm). In this study, a combination of essential oils from *Baeckea frutescens* and *Citrus x microcarpa* Bunge was formulated into two variants, namely Formula 1 (F1) with a ratio of 40:60 and Formula 2 (F2) with a ratio of 60:40. The main objective of this formulation was to improve the physical stability, antibacterial activity, and antioxidant capacity of the two essential oils, which are individually known to have bioactive potential. The choice of nanoemulsion preparation and the type and concentration of surfactant used played an important role in determining the success of the emulsification process, system stability, and droplet size distribution. Based on initial visual observations of the preparations, both formulas showed a homogeneous system without phase separation or sedimentation, indicating that the formation of nanoemulsions was physically successful. Differences in characteristics such as clarity, foam stability, and color between F1 and F2 indicate the influence of the essential oil component ratio on the final properties of the preparation. The results of nanoemulsion formation can be seen in Figure 4 below.



Figure 4. Nanoemulsion formulation exhibiting a homogeneous and visually clear system without turbidity.

Based on figure 4 the visualization results of Formula 1 (F1) and Formula 2 (F2) nanoemulsions, which are combinations of *Baeckea frutescens* and *Citrus x microcarpa* Bunge essential oils, there are differences in physical characteristics. Formula 1 appears cloudier with a dull yellow color and a thin layer of foam, while Formula 2 appears clearer and forms a thicker and more stable layer of foam. This difference in clarity is influenced by the higher proportion of key lime oil in F1, where the content of polar compounds such as flavonoids and organic acids from citrus fruits can cause increased opalescence in the emulsion system. Conversely, the dominance of *Baeckea frutescens* essential oil in F2 tends to produce a cleaner and more stable emulsion due to the lipophilic properties of its terpenoid compounds. The absence of phase separation, flocculation, or sedimentation in both

formulations indicates that these nanoemulsion systems are relatively physically stable during the manufacturing process. Meanwhile, the higher foam characteristics in F2 also indicate a more effective role of surfactants in stabilizing the formulation. The visual differences between the two formulations show that the proportion of essential oil greatly affects the stability and appearance of nanoemulsions.

Particle Size and Polydispersity Index (PDI) Value

Testing the particle size and PI (Polydispersity Index) value of the nanoemulsion formulation using the HORIBA SZ-100 *Particle Size Analyzer* (PSA) instrument aims to determine the particle size and stability of the nanoemulsion formulation. The particle determination data can be seen in Table 4.

Table 4. Measurement Results of Nanoemulsion Formulation Particles of Sapu-sapu Leaf Essential Oil and Essential Oil of Key Lime Fruit Waste

Formulation	Diameter (nm)	PI Value	Particle Size Criteria (nm)
F1	7.06	0.0067	5-200
F2	14.64	0.0368	

Based on the measurement results, the nanoemulsion formulas of sapu-sapu leaf essential oil and key lime waste showed different particle sizes and polydispersity index (PI) values in both formulations. Formula F1 had a particle size of 7.06 nm with a PI value of 0.0067, while formula F2 showed a particle size of 14.64 nm with a PI value of 0.0368. The particle sizes produced from both formulas were within the nano range (<100 nm), which is the main characteristic of nanoemulsion preparations. The smaller particle size in formula F1 indicates that the emulsion system is more capable of producing fine droplets, which can increase the surface area of the particles and accelerate interaction with biological targets such as bacterial cell membranes, thereby potentially increasing antibacterial activity and active ingredient absorption efficiency. Additionally, the very low PI value in F1 (0.0067) indicates that the particle size distribution is highly uniform (monodisperse), ensuring the physical stability of the preparation during storage. Based on this, the F1 formula is superior in terms of physical characteristics, both in terms of smaller particle size and higher particle homogeneity, thus potentially providing more optimal pharmacological effects as an antibacterial and antioxidant preparation.

Antibacterial Activity Test

Antibacterial activity tests were conducted to determine the inhibition zone strength of each nanoemulsion formulation. Antibacterial activity tests in this study were conducted using the disc diffusion method based on bacterial sensitivity to antibacterial agents. The test was performed using nutrient agar media and blank discs that had been soaked in the test and control solutions, then placed on the agar

media and observed for the inhibition zones formed around the discs. The nutrient agar media served as a selective medium for bacterial growth. The discs served to absorb the nanoemulsion preparation and were placed on the bacterial growth medium or agar media. This antibacterial activity test used gram-positive bacteria, *S. aureus* and *S. epidermidis*, and gram-negative bacteria, *E. coli* and *C. acnes*. The antibacterial test results for the formulation are shown in Table 5 as follows:

Table 5. Results of Formulation Bacterial Tests against *S. aureus, S. epidermidis, E. coli* and *C. acnes*

Sample	*E. coli*		*S. aureus*		*S. epidermidis*		*C. acnes*	
	Inhibition zone (mm)	Categori	Inhibition zone (mm)	Categori	Inhibition zone(mm)	Categori	Inhibition zone(mm)	Categori
F1	5.10 ± 0.51	Currently	7.32 ± 0.91	Currently	7.09 ± 0.99	Currently	4.80 ± 1.72	Weak
F2	5.55 ± 0.65	Currently	6.41 ± 0.92	Currently	5.05 ± 1.02	Currently	5.52 ± 1.17	Currently
Positive Control (+)	22.80	Very Strong	34.65	Very Strong	40.48	Very Strong	28.45	Very Strong
Negatif Control (-)	0	-	0	-	0	-	0	-

Based on the data in Table 5, the antibacterial test results show that the nanoemulsion formulation combining essential oils from *Baeckea frutescens* and *Citrus x microcarpa* Bunge has varying levels of activity against four types of pathogenic bacteria, namely *E. coli*, *S. aureus*, *S. epidermidis*, and *C. acnes*. The inhibition zones in Formula 1 (F1) and Formula 2 (F2) ranged from 4.80 mm to 7.32 mm, which is categorized as weak to moderate antibacterial activity. Formula 1 (F1), with an essential oil ratio of *Baeckea frutescens* : *Citrus x microcarpa* of 40:60, provided the highest inhibition zone against *Staphylococcus aureus* (7.32 mm) and *Staphylococcus epidermidis* (7.09 mm), indicating that the active compounds in key lime, such as limonene, citral, and flavonoids, play a dominant role in inhibiting the growth of gram-positive bacteria. Meanwhile, its effectiveness against *C. acnes* was relatively weak (4.80 mm), due to the more complex structure of this bacterium's cell wall and its anaerobic environment, which is more resistant to phenolic compounds.

Formula 2 (F2) with EOB:EOC (60:40) ratio, on the other hand, is more effective against *E. coli* and *C. acnes* than F1, although it is still classified as moderate. The eugenol, cineol, and methyl eugenol compounds in *B. frutescens* contribute to its antibacterial activity against gram-negative bacteria such as *E. coli*. However, the difference in inhibition zones between F1 and F2 is not extremely significant, indicating that the combination of the two essential oils has a fairly broad spectrum of activity against various types of bacteria, but does not reach the very high activity levels seen in the positive control (inhibition zone > 22 mm in all bacteria).

This shows that this nanoemulsion preparation has quite good potential, although it is not as strong as synthetic antibacterial agents. However, as a natural ingredient, this potential remains significant and can be improved through formula optimization or other combinations.

Antioxidant Activity Test

This test was conducted using the DPPH (1,1-diphenyl-2-picrylhydrazil) method to test the ability of a compound to capture free radicals based on the decrease in the intensity of the purple color of DPPH measured with a UV-Vis spectrophotometer at a wavelength of 517 nm Baliyan *et. al.* (2022). Ascorbic acid, employed as a positive control, was evaluated at concentrations of 2, 4, 6, 8, and 10 ppm, demonstrating an IC_{50} value of 7.67 µg/mL. The formulated nanoemulsions were subsequently assessed at five concentration levels: 10, 15, 20, 25, and 30 ppm. The results of absorbance and % inhibition measurements on samples with various concentration variations can be seen in table 6 below:

Table 6. Absorbance and % inhibition of Formulation 1

Concentration (ppm)	Absorbance (Average)	% Inhibition
10	0.415	37.37
15	0.379	42.81
20	0.360	45.67
25	0.338	48.94
30	0.327	50.65
Blanko	0.663	

Based on the data in Table 6, the increase in essential oil concentration is directly proportional to the increase in the percentage of inhibition. Furthermore, the calculation of the % inhibition of each concentration was then plotted in a linear regression graph to determine the IC_{50}. The regression equation obtained is as follows:

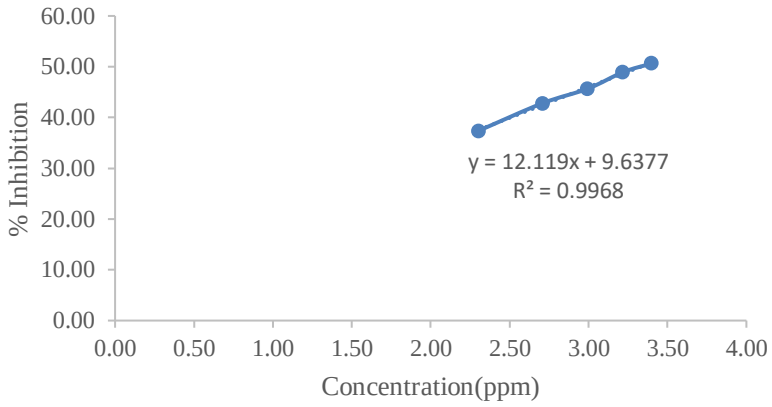


Figure 5. Linear regression curve of Nanoemulsion Formulation 1

Based on the regression curve in Figure 5, the regression equation y = 12.119x + 9.6377 was obtained, with an R^2 value of 0.9968. The IC_{50} calculation is the concentration that causes 50% inhibition, so using the substitution y = 50 with the following calculation:

$$IC_{50} = \frac{50 - 9.6377}{12.119} = \frac{40.3623}{12.119} = 3.3305 \text{ µg/mL}$$

The calculated IC_{50} value of 3.3305 µg/mL indicates very strong antioxidant activity. This value demonstrates a substantial improvement compared to individual essential oils reported in previous studies. Tafdila (2025) documented an IC_{50} value of 135.50 µg/mL for key lime fruit waste essential oil, classified as moderate antioxidant activity. Furthermore, *Baeckea frutescens* L. essential oil exhibited very weak antioxidant activity with an IC_{50} value of 481.525 ± 5.455 µg/mL using the ABTS method (Mahfudh, *et. al*., 2025). The remarkable enhancement in antioxidant activity observed in formula 1 nanoemulsion suggests synergistic interactions between the two essential oils and improved bioavailability of bioactive compounds through nanoemulsification, positioning it as a promising candidate for natural antioxidant applications in pharmaceutical and food industries.

Table 7. Absorbance and % inhibition of Formula 2

Concentration (ppm)	Absorbance (Average)	% Inhibition
10	0.604	24.31
15	0.582	27.07
20	0.546	31.58
25	0.521	34.75
30	0.503	36.93
Blanko	0.798	

Based on the data in Table 7, the increase in essential oil concentration is directly proportional to the increase in the percentage of inhibition. Furthermore, the calculation of the % inhibition of each concentration was then plotted in a linear regression graph to determine the IC_{50}. The regression equation obtained is as follows:

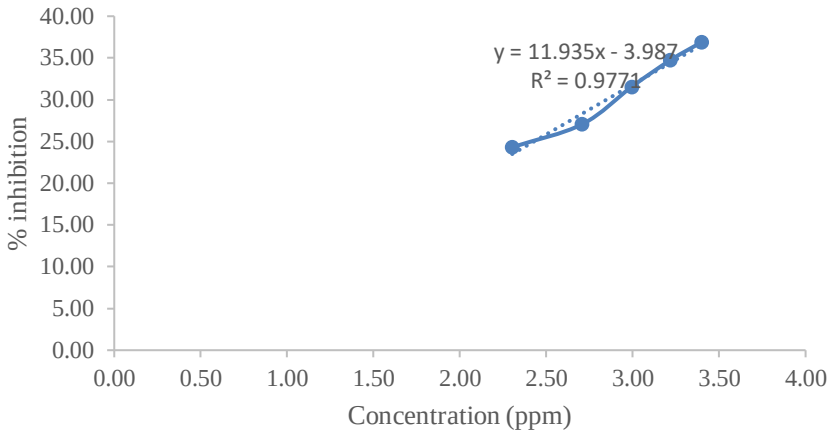


Figure 6. Linear regression curve of Nanoemulsion Formulation 2

Based on the regression curve in Figure 6, the regression equation y = 11.935x - 3.987 was obtained, with an R^2 value of 0.9771. The IC_{50} calculation is the concentration that causes 50% inhibition, so using the substitution y = 50 with the following calculation:

$$IC_{50} = \frac{50+3.987}{11.935} = \frac{53.987}{11.935} = 4.5234 \text{ µg/mL}$$

The calculation shows an IC_{50} value of 4.5234 µg/mL. Based on research data from Tafdila (2025), the combination of nanoemulsions in Formula 2 also shows great potential as a natural antioxidant source because it is classified as a very strong antioxidant.

Based on antioxidant activity testing, it was found that an increase in concentration in nanoemulsions is directly proportional to free radical inhibition strength. This indicates that antioxidant capacity increases with the amount of active compounds added, particularly phenolic compounds and monoterpenoids from essential oil combinations (Khodaei *et. al.*, 2021). The R^2 values obtained from both samples also show that the combination of these samples has a coefficient of determination close to +1 (positive value), which means that there is a good correlation between the sample concentration and the % inhibition (Badawy., 2020). However, there is a significant difference between Formula 1 and Formula 2 in their ability to capture free radicals. Formula 1 showed an IC_{50} value of 3.3305 ppm, while Formula 2 had an IC_{50} value of 4.5234 ppm. The lower IC_{50} value in Formula 1 indicates that this formula is more effective in inhibiting 50% of DPPH free radicals compared to formula 2, because a smaller concentration is needed to achieve the same inhibitory effect.

In terms of classification, both formulas fall into the category of very strong antioxidants, but formula 1 is at a very strong level, indicating higher potential as a natural source of antioxidants. This difference is most likely influenced by the composition of essential oils in each formula, where Formula 1 contains higher

concentrations of active compounds such as phenolics and monoterpenoids (e.g., eugenol, limonene, or terpineol) or works synergistically to enhance antioxidant activity. This indicates that the combination of sapu-sapu leaf essential oil and key lime peel waste in Formula 1 is more optimal in stabilizing free radicals, making it potentially superior for use as an active ingredient in pharmaceutical or natural cosmetic preparations.

Conclusion

Based on the results of the study, it can be concluded that the nanoemulsion preparation combining essential oil from sapu-sapu leaves (*Baeckea frutescens* L.) and key lime waste (*Citrus x microcarpa* Bunge) was successfully formulated with good physical characteristics, marked by small particle size and low Polydispersity Index (PDI) values, indicating the stability and homogeneity of the system. Formulation F1 has a particle size of 7.06 nm with a PDI of 0.0067, while F2 has a size of 14.64 nm with a PDI of 0.1528. Based on antioxidant activity testing, F1 showed an IC_{50} value of 3.3305 ppm, which was lower than F2 at 4.5234 ppm, indicating that F1 has a higher free radical scavenging ability. In addition, both formulations showed antibacterial activity against four types of bacteria, namely *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli*, and *Cutibacterium acnes*. The inhibition zones produced were classified as moderate to strong, with F1 generally providing larger inhibition zones than F2. Based on the results of both tests, The nanoemulsion from the combination of these two essential oils is considered to have the potential to be used as an effective natural and halal-based antioxidant and antibacterial agent in the pharmaceutical or food application fields.

Conflict of Interest

The authors state that the publication of this manuscript involves no conflicts of interest. Furthermore, all ethical standards have been strictly upheld, including those related to plagiarism, informed consent, research misconduct, data fabrication or falsification, duplicate publication or submission, and redundancy.

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