Advances in Healthcare Research
Volume 3, Issue 2 (2025)
Website: https://advancesinresearch.id/index.php/AHR
OPEN ACCESS
e-ISSN: 2985-9808

Formulation and Antibacterial Activity Testing of Face Mist Made from Red Betel Leaf (Piper Crocatum Ruiz and Pav.) Ethanol Extract Against Propionibacterium Acnes

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ARTICLE HISTORY

Received: July 11, 2025
Revised: July 31, 20025
Accepted: August 14, 2025

DOI :
https://doi.org/10.60079/ahr.v3i2.571

ABSTRACT

Purpose: This study aimed to formulate and evaluate the antibacterial activity of face mist containing ethanol extract of red betel leaf (Piper crocatum Ruiz & Pav.) against Propionibacterium acnes, with the hypothesis that higher extract concentrations enhance antibacterial efficacy.

Research Method: A quantitative experimental design was conducted using three formulations with extract concentrations of 15%, 25%, and 35%, alongside positive (clindamycin 300 mg) and negative (base face mist) controls. Samples were obtained from Kayen Village, Pati Regency, Indonesia. Physical evaluations included organoleptic, homogeneity, pH, and spreadability tests, while antibacterial activity was assessed using the disc diffusion method. Data were analyzed with Shapiro–Wilk, Levene's, One-Way ANOVA, and LSD Post Hoc tests.

Results and Discussion: All formulations met physical quality standards. Antibacterial testing showed inhibition zones of 7.57 mm (moderate) for 15%, 10.67 mm (strong) for 25%, and 13.47 mm (strong) for 35%, with higher concentrations producing greater inhibition. Positive control exhibited a 37.60 mm (very strong) inhibition zone, while the negative control showed no activity.

Implications: Red betel leaf extract demonstrates potential as a natural antibacterial ingredient in acne treatment formulations. The 35% concentration was optimal but still less effective than clindamycin. Future research should focus on optimizing formulation stability, enhancing sensory appeal, and exploring synergistic combinations to improve efficacy.

Keywords: piper crocatum; red betel leaf extract; face mist formulation; propionibacterium acnes; antibacterial activity.

Introduction

Red betel (Piper crocatum Ruiz and Pav.) is a medicinal plant with great potential in the treatment of various diseases, as well as high spiritual value. This plant belongs to the Piperaceae family and is known for its shiny silver-red leaves when exposed to light. In the 1990s, red betel began to gain popularity as an ornamental plant due to its attractive appearance. In recent years, this plant has gained increasing attention and is being utilized as a herbal medicine (Rachmawaty *et al.*, 2021). Based on

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existing research, red betel has numerous benefits beyond its antibacterial properties, including anti-inflammatory, anti-itch, cough-relieving, antiseptic, and hemostatic effects (Hermanto *et al.,* 2023). Red betel leaves are one of the traditional plants proven effective in treating acne. Red betel leaves also have antibacterial properties against Propionibacterium acnes, the bacteria responsible for acne (Afidhah, 2022). Red betel leaves contain chemical compounds such as alkaloids, flavonoids, tannins, and essential oils, which are believed to have antimicrobial potential. Red betel leaves are typically extracted using ethanol to enhance the effectiveness of the antimicrobial compounds they contain. The polyphenols in red betel possess antimicrobial activity, as they can disrupt bacterial enzymes and inhibit protein synthesis on the bacterial cell surface (Rachmawaty *et al.,* 2021). Red betel leaves (Piper crocatum Ruiz and Pav.) contain several main compounds that play a role in acne treatment, including alkaloids, flavonoids, tannins, and essential oils. The specific proportions of each of these compounds have not been thoroughly investigated and require further research. Several studies have identified the main components in red betel leaf essential oil. One study found that the essential oil content in red betel leaves reached approximately 4.2%, with the main compounds being β-phenol, eugenol, and cavicol (Andayani *et al.,* 2020).

Acne is one of the most common skin infections, particularly among teenagers. Acne, also known as acne vulgaris, is a chronic inflammatory condition that affects the pilosebaceous unit. This condition can resolve on its own and presents various clinical manifestations, including comedones, papules, pustules, nodules, and scar tissue; hence, it is often referred to as a pleomorphic skin disease. In addition to hormonal factors and follicle blockage, acne is usually exacerbated by bacterial infection caused by Propionibacterium acnes, which attacks inflamed skin tissue. These bacteria play a role in the formation of pus. The mechanism of acne development involves stimulation of sebaceous glands, leading to excessive sebum production, typically beginning during puberty. Abnormal keratinocyte proliferation, as well as adhesion and differentiation of the lower branches of the follicle, also contribute to the formation of inflammatory lesions associated with Propionibacterium acnes. Acne treatment aims to improve abnormal follicles, reduce sebum production, suppress the number of P. acnes colonies or their metabolic products, and reduce skin inflammation (Sifatullah & Zulkarnain, 2021). The highest prevalence of acne occurs between the ages of 14 and 17, with approximately 83–85% of women and 95–100% of men aged 16–19 being affected. In women, acne may occasionally persist until around age 30, while this is rare in men. Acne has a significant impact on the lives of those affected, primarily because it often appears on the face, making it difficult to conceal. Although it may resolve on its own in some cases, the condition typically progresses or persists over an extended period with varying degrees of severity. Many individuals with acne experience difficulties in social interactions. Depression is the most common psychological change, even in cases of mild to moderate acne. The severity of acne varies from mild to severe and can affect mood, self-confidence, and quality of life (Imas Roro Ayu Sekar Tyasari, Siti Maisyaroh Bakti Pertiwi, 2022).

Facial skin health requires regular care, including the use of cosmetics. Cosmetics are products applied to the body to cleanse, beautify, provide fragrance, or alter appearance, but they are not permitted to affect the user's health. Herbal extracts generally possess various functions due to their beneficial properties, including protection against UV rays, anti-aging effects, moisturizing, astringent, anti-irritant, and antimicrobial properties that are interrelated (Rahmawati *et al.,* 2024). One form of natural cosmetic that can be used is face mist (Widyasanti & Fauziyah, 2022). Face mist is a beauty care product in spray form that functions to increase the moisture of the outer layer of the skin. The use of face mist offers several benefits, including refreshing the skin, moisturizing it, and creating a protective

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coating. Additionally, face mist can enhance the effectiveness of creams, toners, and other skincare products. This face mist formulation is efficient and easy to apply, as it can be sprayed directly onto the face without needing to be used with hands, thereby reducing contact between the hands and the facial skin. Furthermore, face mist has the advantage of faster absorption compared to other formulations (Sakka & Hasma, 2023).

Various formulations have been developed for face mist preparations in previous studies; however, none of the earlier studies have developed a face mist based on compounds from red betel leaf (Piper crocatum Ruiz and Pav.). This study aims to evaluate the antibacterial activity of a face mist formulated from red betel leaf ethanol extract against Propionibacterium acnes, thereby enabling the product to serve as a natural alternative in acne treatment. The results of this study are expected to contribute to the development of safer and more effective skin care products. Based on the above, the researcher conducted a study titled "Formulation & Antibacterial Activity Testing of Face Mist Made from Ethanol Extract of Red Betel Leaf (Piper Crocatum Ruiz and Pav.) Against Propionibacterium Acnes."

Literature Review and Hypothesis Development

Mechanism of Action of Propionibacterium acnes

The concentration of ethanol extract from betel leaves is expected to influence the effectiveness of face mist in inhibiting or killing Propionibacterium acnes. Propionibacterium acnes is a Gram-positive anaerobic bacterium that is the primary inhabitant of the human skin microbiota and dominates the pilosebaceous unit (Dalope *et al.,* 2024). This bacterium is also capable of producing propionic acid (Rindi Novitri Antika, 2020). Red betel leaf ethanol extract was prepared to optimize the content of its antibacterial active compounds. Red betel leaf ethanol extract can also inhibit bacterial growth due to its active compounds, including flavonoids, tannins, alkaloids, and saponins, which exhibit antibacterial activity (Iwani *et al.,* 2024).

Mechanism of Action of Compounds in Red Betel Leaves

The mechanism of action of saponins functions as an antibacterial agent by damaging the cytoplasmic membrane, ultimately leading to bacterial cell death (Triyani *et al.,* 2021). The mechanism of action of flavonoids works by inhibiting DNA gyrase and ATPase enzymes, thereby disrupting bacterial growth processes. Alkaloids exhibit antibacterial activity, which is believed to occur through a mechanism that disrupts peptidoglycan, a significant component of the bacterial cell wall, thereby preventing the cell wall from forming properly and leading to cell death. The mechanism of action of saponins involves forming complexes with cell membranes through hydrogen bonding, which disrupts cell wall permeability and triggers cell death. The mechanism of action of tannins acts as an antibacterial agent by forming complexes with bacterial cell wall polysaccharides, disrupting metabolism, and ultimately causing bacterial death (Theresia Avilla Nor, Desi Indriarini, 2020).

Research Method

This study is a quantitative research, which is a method of collecting information that relies on numerical data to test our research questions. Research that relies on numerical data is known as quantitative research (Ummul *et al.,* 2022). In this quantitative study, the author aims to determine the optimal formulation and testing method for red betel leaf (Piper crocatum Ruiz and Pav.) ethanol extract as a face mist. This study was conducted at the Pharmacy Laboratory at Muhammadiyah University

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Kudus and the Pharmacy Laboratory at the Cendekia Utama Kudus Health Technology Institute (ITEKES). The population used in this study was 10 kg of red betel leaves obtained from Kayen Village, Kayen District, Pati Regency. Red betel leaves have heart-shaped leaves with a pointed tip, measuring 15–20 cm in length and 3.5–10 cm in width. The upper surface of the leaves is dark green with white-grayish patterns, while the lower surface is reddish-purple or bright red. The leaf surface is glossy and smooth, without hairs. The sample used was 1 kg of red betel leaves, which were then made into an extract. This study used three concentrations: 15%, 25%, and 35%, each of which was tested for bacterial activity. The samples were obtained from Kayen District, Pati Regency. The data collection technique used in this study was observation. Data analysis in this study is presented in tabular form to compare the differences in antibacterial activity at each concentration of 15%, 25%, and 35%. After the data passed the normality and homogeneity tests to determine whether there were differences in treatment or whether the average of each treatment yielded different results, the ANOVA Post Hoc test was used. Using statistical calculations with One-Way ANOVA ($p<0.005$), the differences in antibacterial activity at each concentration (15%, 25%, and 35%) were compared.

Results and Discussion

Analysis Result

Plant Identification Results

The results of plant identification conducted at the Biology Laboratory of Ahmad Dahlan University in Yogyakarta indicate that the test plant used in this study is red betel leaf (Piper crocatum Ruiz and Pav.).

The following are the results of plant identification:

1b – 2b – 3b – 4b – 12b – 13 b – 14b – 17b – 18b – 19b – 21b – 22b -23b – 24b – 25b – 26b – 27b – 799b – 800b – 801b – 802a – 803b – 804b – 805c – 806b – 807a – 808c – 809b – 810b – 811a – 812b – 815b – 816b – 818b – 820b – 821b – 822a – 823b Piperaceae
1b – 2b – 3b Piper

Piper crocatum Ruiz & Pav. Flora of Java (Backer,1965)

Sample Processing Results

The processing of red betel leaf simplisia was carried out using a drying process under sunlight covered with black cloth. The results of the sample processing are presented in Table 1.

Table 1. Preparation of Red Betel Leaf Simplisia

Parameters	Results
Fresh Leaf Weight (grams)	10000 grams
Weight of Dried Simplisia (grams)	2500 grams
Drying Shrinkage (%)	75 %
Water Content	2,67%

Based on the data in Table 1, the fresh leaf weight was 10,000 grams. After the drying process, the dry weight of the crude drug was 2,500 grams. This indicates a drying loss of 75%, indicating that most of the water content evaporated during the process. The moisture content remaining in the dried

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simplisia was recorded at 2.67%, indicating that the material has undergone a sufficiently optimal drying process.

Extract Yield Results

Red betel leaf extract (Piper crocatum Ruiz and Pav) was obtained from red betel leaves that were harvested, dried, and processed in a blender to obtain 1000 grams of red betel leaf powder. The extraction process employs the maceration method, using 70% ethanol as the solvent, with a ratio of crude extract to solvent of 1:10. This is illustrated in Table 2.

Table 2. Yield of Red Betel Leaf Extract

Sample	Initial weight	Final weight	Results
Red Betel Leaf Powder (Piper crocatum Ruiz and Pav.)	1000 gram	102,17 gram	9,78%

Based on the data in Table 2, red betel leaf powder (Piper crocatum Ruiz and Pav.) with an initial weight of 1,000 grams underwent shrinkage to reach a final weight of 102.17 grams after a specific process. The results of this process indicate that the yield obtained was 9.78%, reflecting the extraction or processing efficiency of the raw material into the final product, which is dry powder.

Ethanol-Free Test Results

The concentrated extract of red betel leaves was then evaluated for ethanol removal to obtain a clean extract free of ethanol contamination. The findings from the evaluation of the ethanol-free red betel leaf extract indicated that the extract was free of ethanol, as evidenced by the absence of an ether-like odor during the review. This is illustrated in Table 3.

Table 3. Ethanol-free test of red betel extract

Identification	Reagent	Results
Ethanol-Free Test	H2SO4 + CH3COOH	No smell of ether

Phytochemical Screening Results

The red betel leaf extract obtained was then identified for its chemical compounds. The results of the chemical compound identification test showed that the red betel leaf extract contains alkaloids, flavonoids, tannins, and saponins. The phytochemical test presented in Table 4 shows the presence of several categories of active chemicals in the sample. The alkaloid test, using three reagents —Mayer, Dragendorff, and Wagner —produced positive results for alkaloid compounds, as evidenced by the formation of white, orange, and brown precipitates. In addition, experiments conducted on flavonoid compounds using magnesium powder and concentrated hydrochloric acid (HCl) showed a color transition to orange, indicating the presence of flavonoids. The saponin test with distilled water produced persistent foam, indicating a positive result for saponin chemicals. The tannin compound test using 1% $FeCl_3$ produced a blackish-green color, confirming the presence of tannin compounds in the sample. As a result, the sample was found to contain alkaloids, flavonoids, saponins, and tannins.

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Table 4. Phytochemical Screening Results

Compound Groups	Reagen	Before	Results	Conclusion
Alkaloid	Mayer	Dark green	White Sediment	Positive alkaloid
	Dragendrof	Dark green	Orange sediment	Positive alkaloid
	Wagner	Dark green	Brown Sediment	Positive alkaloid
Flavonoid	Magnesium powder and concentrated HCl	Dark green	Orange color	Positive flavonoids
Saponin	Aquadest	Dark green	The foam doesn't disappear	Positive saponin
Tanin	FeCl3 1%	Dark green	Dark green	Positive saponin

Face Mist Formulation Results

The positive control in this study used clindamycin 300 mg, while the negative control used a face mist base. Formulation 1 (F1) was a face mist preparation containing 15% extract, Formulation 2 (F2) was a face mist preparation containing 25% extract, and Formulation 3 (F3) was a face mist preparation containing 35% extract.

Table 5. Formula Design

Material	Function	Formulation				
		K(+)	F(0)	F1	F2	F3
Clindamycin	Control	1	-	-	-	-
Red betel leaf ethanol extract	Active ingredient	-	-	15	25	35
Gliserin	Emollient	-	20			
PVP	Additional materials	-	4	4	4	4
Metilparaben	Preservative	-	0,02	0,02	0,02	0,02
Aquadest	Solvent	-	ad 100 ml	ad 100 ml	ad 100 ml	ad 100 ml

Source: (Wahyuningsih et al., 2023).

Face Mist Preparation Evaluation Test Results

The evaluation test of face mist preparations involves several observations, including organoleptic assessments, homogeneity evaluations, pH value measurements, and spreadability measurements. The evaluation test was conducted on three face mist formulations with different extract (active ingredient) concentrations.

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Website: https://advancesinresearch.id/index.php/AHR
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e-ISSN: 2985-9808

Organoleptic Observation Test Results

Table 6. Results of Organoleptic Observation Test of Extracts

Extract	Observation		
	Color	Smell	Shape
Red Betel Leaf	Dark Green	Characteristic odor of the extract	Thick

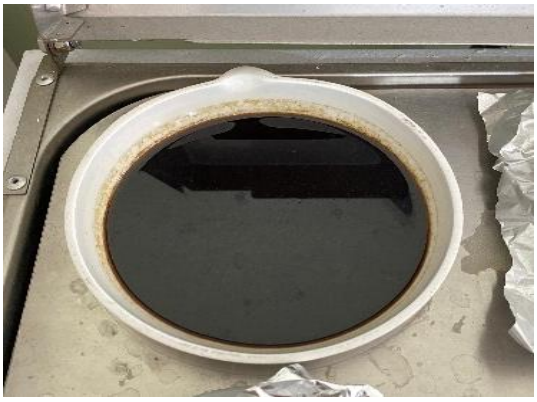

Figure 1. Extract of red betel leaves

Based on the observation results, the red betel leaf extract exhibited physical characteristics in the form of a blackish-green color, accompanied by a strong characteristic extract odor. The extract appeared thick, which is a common characteristic of concentrated extracts resulting from the extraction process of active compounds from plant materials. These characteristics indicate that the extract has undergone a good concentration process and is ready for use in further formulation.

Table 7. Results of Organoleptic Observation Test of Face Mist

Formulation	Observation		
	Color	Smell	Shape
F0 (K-)	Clear	Odorless	Liquid
F1 (15%)	Dark Green	Characteristic odor of the extract	Liquid
F2 (25%)	Dark Green	Characteristic odor of the extract	Liquid
F3 (35%)	Dark Green	Characteristic odor of the extract	Liquid

Based on the results of the observations in Table 7, various formulations exhibited differences in physical characteristics between the negative control formula (F0) and those containing red betel leaf extract (F1, F2, and F3). Formula F0 (negative control) was clear, odorless, and liquid. Meanwhile, formulas F1 (15%), F2 (25%), and F3 (35%) exhibit a dark green color, a characteristic odor of the extract, and remain in a liquid form. The changes in color and odor from F1 to F3 reflect the addition of extract in the formulation, while the liquid consistency remains consistent across all formulations.

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OPEN ACCESS
e-ISSN: 2985-9808

Homogeneity Observation Test Results

Based on the results of the evaluation of the homogeneity of the preparations in Table 8, all formulations —namely, F0 (negative control), F1 (15%), F2 (25%), and F3 (35%) —showed homogeneous properties. These observations are made by the specified homogeneity requirements, so all formulations are declared to meet these requirements. Good homogeneity indicates that all components in the formulation are evenly mixed, which is crucial for ensuring the stability and efficacy of the final product.

Table 8. Results of Homogeneity Observation Test

Formulation	Observation	Requirements	Description
F0 (K-)	Homogen	Homogen	Eligible
F1 (15%)	Homogen	Homogen	Eligible
F2 (25%)	Homogen	Homogen	Eligible
F3 (35%)	Homogen	Homogen	Eligible

Results of pH Value Observations

Table 9. pH Observation Results

Formulation	Observation				
	Replication 1	Replication 2	Replication 3	$\bar{x}$ ± SD	Description
F0 (K-)	3,46	3,42	3,68	3,52 ± 0,14	Not eligible
F1 (15%)	5,05	5,29	5,32	5,22 ± 0,15	Eligible
F2 (25%)	5,27	5,28	5,29	5,28 ± 0,001	Eligible
F3 (35%)	5,24	5,22	5,24	5,23 ± 0,001	Eligible

Based on the observation data in Table 9, for the four formulations (F0 to F3), it was found that the average observation value for F0 (negative control) was 3.52 and did not meet the specified requirements. Conversely, formulations with the addition of active ingredients starting from F1 (15%), F2 (25%), and F3 (35%) showed average values above 5, namely 5.22, 5.28, and 5.23, respectively, all of which meet the criteria. This indicates that the addition of active ingredients has a positive effect on the observed parameters, where higher percentages of active ingredients result in increasing observation values up to the optimal limit.

Dispersion Power Measurement Test Results

Table 10. Results of Power Distribution Measurement Test

Formulation	Observation				
	Replication 1	Replication 2	Replication 3	$\bar{x}$ ± SD	Description
F0 (K-)	5,40	5,60	5,70	5,57 ± 0,15	Eligible
F1 (15%)	5,2	5,5	5,3	5,33 ± 0,15	Eligible
F2 (25%)	6,3	5,4	5	5,57 ± 0,66	Eligible
F3 (35%)	6	5,8	5	5,60 ± 0,52	Eligible

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e-ISSN: 2985-9808

Based on the results of the observations in Table 10, all formulations (F0 to F3) showed average values within the range of 5 to 7, thus meeting the established quality requirements. Formulation F0 (control) has the highest average value of 5.56, followed by F1 (15%) at 5.33, F2 (25%) at 5.28, and F3 (35%) at 5.23. Despite variations in values between replicates, all formulations still meet the expected quality criteria. This indicates that increasing the active ingredient content up to 35% does not reduce product quality below the specified standards.

Bacterial Identification Results (Gram Stain)

Table 11. Identification of bacteria

Types of bacteria	Treatment/ Reagen	Observation	Results
Propionibacterium acnes	Crystal violet Iodine Safranin Aquadest	Basil-shaped, purple	Positive for Propionibacterium acnes bacteria

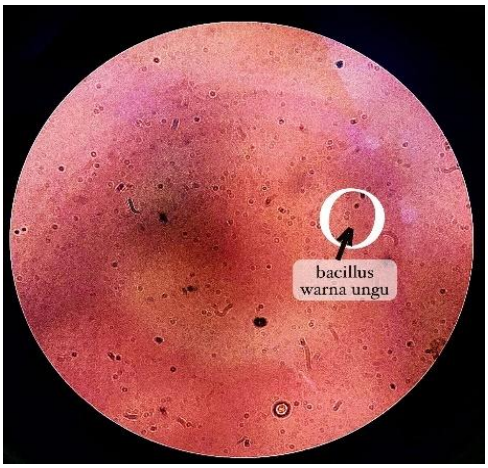

Figure 2. Propionibacterium acnes

Testing of Propionibacterium acnes bacteria using Gram staining with crystal violet, iodine, safranin, and aquadest reagents showed positive results. Based on microscopic observation, the bacteria appeared bacillus-shaped (rod-shaped) and purple, indicating that P. acnes is a Gram-positive bacterium. These results confirm the presence of Propionibacterium acnes in the tested sample.

Results of Antibacterial Activity Test of Face Mist Preparation from Red Betel Leaf Extract (Piper crocatum Ruiz and Pav.)

The prepared formulations were then tested for antibacterial activity against Propionibacterium acnes bacteria using the disc diffusion method. Formulation 0 did not contain any active ingredients and served as a negative control. Formulation F1 contained 15% active ingredient extract, formulation F2 contained 25% active ingredient extract, formulation F3 contained 35% active ingredient extract, and clindamycin was used as the positive control in the antibacterial test.

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Table 12. Results of Observation of the Inhibition Zone

Formulation	Observation of the Barrier Zone (mm)				
	Replication 1	Replication 2	Replication 3	x̄ ± SD	Description
K (+)	3,7	39,5	36,5	37,60 ± 1,61	Very strong
K (-)	0	0	0	0 ± 0	-
F1 (15%)	7,3	7,5	7,9	7,57 ± 0,31	Moderate
F2 (25%)	9,5	10,5	12	10,67 ± 1,26	Strong
F3 (35%)	13	13,5	13,9	13,47 ± 0,45	Strong

The zone inhibition test results showed that the positive control (K+) exhibited potent inhibition, with an average measurement of 37.60 mm and a standard deviation of 1.61 mm. The negative control (K−) showed no antibacterial activity, with an average zone inhibition of 0 mm and a standard deviation of 0 mm. Formulation F1 (15%) showed moderate efficacy, with an average inhibition zone of 7.57 mm and a standard deviation of 0.31 mm, reflecting somewhat consistent results. Formulation F2 (25%) and F3 (35%) showed significant activity, with average inhibition zones of 10.67 mm (SD 1.26) and 13.47 mm (SD 0.45), respectively. Higher extract concentrations were correlated with larger inhibition zones, indicating increased antibacterial efficacy, particularly against Propionibacterium acnes.

Normality Test

The Normality Test aims to see whether the data is normally distributed. The Normality Test using Shapiro-Wilk shows significant results if the Sig. value is > 0.05. The results are presented in Table 13.

Table 13. Normality Test Results

		Kolmogorov-Smirnova			Shapiro-Wilk		
	Data	Statistic	df	Sig.	Statistic	df	Sig.
Antibacterial	F1	.253	3	.	.964	3	.637
	F2	.219	3	.	.987	3	.780
	F3	.196	3	.	.996	3	.878
	Kontrol Positif	.328	3	.	.871	3	.298
a. Lilliefors Significance Correction							
Significance value > 0,05							

Homogeneity Test

Table 14. Homogeneity Test Results

		Levene Statistic	df1	df2	Sig.
Antibacterial	Based on Mean	3.334	3	8	.077
	Based on Median	.759	3	8	.548
	Based on Median and with adjusted df	.759	3	3.502	.579
	Based on the trimmed mean	3.055	3	8	.092
Significance value > 0,05					

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The homogeneity test aims to determine whether the variance of data between different groups is the same or not. The results of the homogeneity test using Levene's Statistic show significant results if the Sig. value is > 0.05.

Significance value > 0,05

One-way ANOVA Test

The one-way ANOVA test aims to determine whether there are significant differences between group averages. The one-way ANOVA test shows significant results if the Sig. value is < 0.05. The results are presented in Table 15.

Table 15. Results of One-way ANOVA Test

Antibacterial

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1704.682	3	568.227	509.240	.000
Within Groups	8.927	8	1.116		
Total	1713.609	11			

Significance value < 0,05

Table 16. Post Hoc Tests

Multiple Comparisons
Dependent Variable: Antibakteri
LSD

(I) Data	(J) Data	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F1	F2	-3.10000*	.86249	*.007	-5.0889	-1.1111
	F3	-5.90000*	.86249	*.000	-7.8889	-3.9111
	Positive Control	-30.10000*	.86249	*.000	-32.0889	-28.1111
F2	F1	3.10000*	.86249	*.007	1.1111	5.0889
	F3	-2.80000*	.86249	*.012	-4.7889	-.8111
	Positive Control	-27.00000*	.86249	*.000	-28.9889	-25.0111
F3	F1	5.90000*	.86249	*.000	3.9111	7.8889
	F2	2.80000*	.86249	*.012	.8111	4.7889
	Positive Control	-24.20000*	.86249	*.000	-26.1889	-22.2111
Positive Control	F1	30.10000*	.86249	*.000	28.1111	32.0889
	F2	27.00000*	.86249	*.000	25.0111	28.9889
	F3	24.20000*	.86249	*.000	22.2111	26.1889

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

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Post Hoc Test

A post hoc test was conducted to identify sample groups with similar and dissimilar average antibacterial activities. If the significance value was > 0.05, it could be concluded that the sample groups had identical average antibacterial activities. If the significance value was < 0.05, it could be concluded that the sample groups had dissimilar average antibacterial activities.

Discussion

Plant Determination

The plants selected for this study were first identified to verify the authenticity of the specimens used in the study. Identification was carried out by correlating the morphological characteristics of the plants with the identification key (Klau & Hesturini, 2021). The validity of the plants used in this study was confirmed by a certificate from the Biology Laboratory of Ahmad Dahlan University, Yogyakarta, No. 067/Lab.Bio/B/I/2025, stating that the plants used were red betel leaves (Piper crocatum Ruiz and Pav.).

Processing of Red Betel Leaves (Piper crocatum Ruiz and Pav.)

Red betel leaves from Kayen Village, Pati Regency, Central Java, are processed into simplisia through several stages, namely wet sorting to clean the leaves, washing, cutting to accelerate drying, drying under the sun with black cloth protection, dry sorting to remove impurities, and finally grinding with a blender to produce fine and uniform simplisia powder. Dried herbal medicine weighing 2,500 grams is made from 10,000 grams of dried red betel leaves, as shown in Table 1. The drying loss of red betel leaves is 75%. A non-specific measurement known as drying loss is used to determine the upper limit or distance at which a compound can be lost after drying. Drying at 105 °C for 30 minutes until the weight reaches stability is the basic method for measuring the remaining substance. The results are presented in percentage form (Mewar, 2023). The measurement parameter for determining the amount of air remaining after drying the crude drug is by testing the air content in the powder. According to the Ministry of Health of the Republic of Indonesia (2014), the moisture content in powdered crude drugs must be less than 10%. To determine the moisture content in red betel leaves, a three-stage moisture balance was used. The moisture content test results were less than 10% for the first red betel leaf (2.51%), the second red betel leaf (2.21%), and the third red betel leaf (3.27%), indicating that the herbal material meets the specified standard. Based on these results, it can be concluded that the crude drug used meets the required moisture content specifications. The growth of bacteria causing inflammation can be accelerated if the moisture content in the crude drug exceeds 10%. Since air is a breeding ground for bacteria, the quality of the crude drug may deteriorate over time (Jayadi, 2022).

Extraction of Red Betel Leaf (Piper crocatum Ruiz and Pav.)

The extract used in this study was red betel leaf extract. The extraction process was carried out using the maceration method with 70% ethanol as the solvent. This method was chosen because it is straightforward to perform. Maceration was carried out using a ratio of 1:10 w/v, with 1000 grams of crude drug and 10 liters of 70% ethanol. After maceration, the filtered filtrate was evaporated. The solvent evaporation process was carried out using a rotary evaporator at a temperature of 50°C with a rotation speed of 60 rpm until a concentrated red betel leaf extract was formed. A temperature of 50°C

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was chosen because under vacuum conditions, ethanol evaporates easily, making the evaporation process fast and efficient. The principle of the rotary evaporator is based on the evaporation of the solvent below its boiling point, where the boiling point of ethanol ranges from 78 °C to 60 °C. With reduced pressure, evaporation occurs at temperatures below 60°C, allowing the solvent vapor to condense and collect in the condenser. This method ensures that the compounds separated from ethanol are not damaged. The extract is then concentrated again using a water bath at 50°C.

Table 2 shows that after maceration and concentration in a water bath, a concentrated extract weighing 102.17 grams was obtained, corresponding to a yield of 9.78%. Thus, the results indicate that the yield of red betel leaf extract is satisfactory. For an extract to be considered concentrated, its yield must be at least 10% (Herbal Pharmacopoeia, 2017). A 2022 study by Rahmaningtyas found that the yield of red betel leaf extract was 15.2% (Rahmaningtyas, 2022). A high yield indicates a larger amount of extract obtained from the plant extraction process; this is a comparison of the amount of extract. The yield value also shows the concentration of bioactive chemical compounds in the extract. The higher the yield, the more extract is produced (Rosa et al., 2023). According to Eka Kusuma (2022), several variables can influence the results of the extraction process. These include the concentration of other solvents, extraction duration, the ratio of raw material to solvent, the combination of solvent concentration and extraction time, solvent concentration, and the ratio of raw material to solvent, as well as the final result.

Ethanol-Free Test

The ethanol-free test ensures that the extract contains no residual ethanol, thereby yielding a pure extract free from contamination. This is important because ethanol has antibacterial and antifungal properties, which can cause false positive results when testing samples (Setiyanto *et al.,* 2024). The ethanol-free test was conducted by adding concentrated acetic acid and concentrated sulfuric acid as reagents and catalysts for esterification to the red betel leaf extract, which was then heated. The ethanol-free test of red betel leaf extract showed no characteristic ester aroma (fragrance). This occurs because no chemical bonds form between the hydroxyl (OH) atoms in acetic acid and the hydrogen atoms in ethanol. The presence of sulfuric acid, which acts as a catalyst and a strong acid, facilitates this reaction, thereby preventing the formation of esters and the resulting odor (Setiyanto *et al.,* 2024). Red betel leaf extract does not contain ethanol, as indicated by the test results in Table 3.

Phytochemical Screening

Phytochemical screening tests were conducted to identify secondary metabolites in red betel leaf extract by adding several chemicals, allowing for their identification through changes in the color of the samples. In this study, four types of screening were conducted, namely alkaloids, flavonoids, saponins, and tannins.

Alkaloid Compound Classification Test

Alkaloid testing was performed using three types of reagents: Mayer's, Dragendorff's, and Wagner's. These three reagents work by forming insoluble complexes between the reagents and the basic groups in alkaloid compounds. In the Mayer test, a white precipitate forms, indicating the presence of alkaloids. The Mayer reagent consists of a solution of mercury (II) chloride and potassium iodide, which reacts with alkaloids to form a mercury-alkaloid precipitate. In the Dragendorff test, an orange-

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brown precipitate appears, indicating a positive result for the presence of alkaloids. The Dragendorff reagent works by forming an insoluble alkaloid-bismuth-iodide complex. In the Wagner test, a brown precipitate forms, indicating a positive result for alkaloids. The Wagner reagent contains iodine and potassium iodide, which form an iodo-alkaloid complex. The three positive results from these reagents confirm the presence of alkaloids in the tested red betel leaf extract. Alkaloids are known to possess various pharmacological activities, such as antibacterial, analgesic, anticancer, and antifungal properties (Harborne, 1987).

Flavonoid Compound Classification Test

The presence of flavonoid components in red betel leaf extract was determined using a flavonoid test. As part of this procedure, the extract sample was treated with magnesium and concentrated hydrochloric acid to produce Wilstater reagent. When tested for flavonoids, a positive result is observed if the color is red, yellow, or orange (Tutik *et al.,* 2021). The reduction of flavonoid components by magnesium and hydrochloric acid causes a color change from red to yellow or orange (Jayadi, 2022).

Saponin Compound Classification Test

The saponin test was conducted to determine whether red betel leaf extract contains saponin compounds. In this study, the saponin phytochemical test was performed using a 1:1 ratio of extract to water, and the mixture was stirred for 1 minute. If stable foam formed for 10 seconds, it indicated that saponin was successfully formed (Adjeng *et al.,* 2020). Saponins produce foam due to their biphasic nature, meaning they are soluble in both water and nonpolar solvents, as well as in air (Jayadi, 2022). Because they contain surface-active components similar to those found in detergents, saponins exhibit antibacterial properties.

Tannin Compound Classification Test

Tannin testing was conducted to detect the presence of tannin compounds in red betel leaf extract. In this study, the tannin test used a reaction with 1% FeCl3, where the extract was mixed with a 1% FeCl3 solution. If the extract contained tannin, a color change to dark green or dark blue would be observed (Amal *et al.,* 2023). This color change occurs due to the reaction between $FeCl_3$ and the hydroxyl groups in the tannin compounds. The color change indicates the presence of condensed tannins (Jayadi, 2022).

Face Mist Product Evaluation

The objective of this study was to evaluate the stability of face mist containing red betel leaf extract (Piper crocatum Ruiz & Pav.). Red betel leaves were selected based on their secondary metabolites, which include anti-inflammatory, antioxidant, and bactericidal essential oils, tannins, and flavonoids (Rachmawaty *et al.,* 2021).

The evaluation was conducted through several physical tests, including organoleptic testing, homogeneity testing, pH testing, and spray spreadability testing.

- Organoleptic Test

  In the organoleptic test, Formulation (0) yielded a clear, odorless, and liquid product. The formulation (15%) resulted in a dark green color, a characteristic extract odor, and a fluid

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consistency. The formulation (25%) resulted in a dark green color, a characteristic extract odor, and a liquid consistency.

The formulation (35%) exhibited a dark green color, a characteristic odor of the extract, and a liquid form, as shown in Table 6. The color change in the formulation is due to the active ingredient, namely red betel leaf ethanol extract. The difference in color intensity is caused by variations in the amount of active ingredient added to each formulation.

The higher the concentration of the active ingredient, the darker the formulation's color will be. The consistency of color, aroma, and physical form in each observation indicates that the face mist formulation remains stable organoleptically during the testing period. A good organoleptic appearance is crucial, as it significantly influences consumer acceptance of cosmetic products (Wahyuningsih *et al.,* 2023). The dark color of the face mist, containing red betel leaf extract, is a natural phenomenon that reflects the high content of active ingredients from the plant. Red betel leaf (Piper crocatum Ruiz & Pav.) contains compounds such as anthocyanins, flavonoids, tannins, and various natural antioxidants, which impart a dark base color, such as a reddish-brown hue. When these compounds are extracted, especially in high concentrations, the final product tends to have a more intense color. This dark color is not a flaw, but rather an indication that the face mist uses natural ingredients without bleaching or the addition of artificial colorants. However, an overly dark color may pose limitations in this study, as it could potentially affect the visual appeal and consumer preferences for cosmetic products. Therefore, in future research, it is recommended to explore color stabilization methods, such as encapsulation, selection of alternative solvents, or adjustment of extract concentrations, to produce formulations with more aesthetically pleasing colors without compromising the efficacy of the natural active ingredients.

- Homogeneity Test

  According to Hidayah et al. (2023), face mist containing silica can be tested for homogeneity by observing it under a microscope for visible particles or sharp edges. With this test, we can ensure that the product contains the correct balance of active and inert components. One advantage of homogeneity is ensuring that active ingredients are consistently delivered to all accessible volumes. This keeps the face mist effective and extends its shelf life. Additionally, uniformity demonstrates that the formulation process has successfully broken down the active and inert ingredients. Good homogeneity, defined as the absence of clumping, separation, or phase separation during application, was demonstrated by all red betel leaf ethanol extract face formulas, according to the results published in Table 8. Therefore, it is safe to conclude that the face mist in question meets the requirements for homogeneity.

- pH test

  The pH measurement of the face mist containing red betel leaf ethanol extract was performed by placing the sample in a beaker and then dipping the pH meter into it (Wahyuningsih *et al.,* 2023). Based on the data in Table 4.7, the average pH values for each face mist formulation are as follows: Formulation (0) has an average pH of 3.53; Formulation 2 (15%) is 5.22; Formulation 2 (25%) is 5.28; and Formulation (35%) is 5.23. According to Wahyuningsih et al. (2023), the pH of face mist formulations should align with the skin's pH range, which is between 4.5 and 6.5. Factors that cause pH instability include temperature and exposure to light, which can impact the stability and quality of a formulation. However, all formulations were considered stable as the pH values remained within the normal skin pH range (Safitri *et al.,* 2025).

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The appropriate pH is crucial for maintaining skin microflora balance and reducing the risk of irritation. Additionally, pH stability during storage indicates the chemical stability of the face mist formulation. From the test results, Formulation (0) did not meet the expected pH criteria, likely due to the initial formulation's ingredient composition not being optimized. Imbalances in the ratios of humectants, active ingredients, preservatives, or solvents can render the formulation's pH unsuitable. Meanwhile, Formulations (15%), (25%), and (35%) met the skin pH standards, indicating that the face mist formulation with red betel leaf ethanol extract meets the required pH requirements.

- Dispersion Test

  In this experiment, the preparation was sprayed onto the surface of plastic mica from a distance of 5 cm, and the spread area was measured using a ruler. Here, the spray diameter serves as the metric. According to Wahyuningsih et al. (2023), adequate spray power is defined as 5 to 7 cm. Table 4.10 shows the average spray spread power values for each formulation in this study. The values range from 5.56 cm for Formula (0) to 5.33 cm for Formula 1, 5.28 cm for Formula 2, and 5.23 cm for Formula 3. The average spray spread of the red betel leaf ethanol extract face mist tends to decrease with increasing extract concentration. However, the spray dispersion requirement for facial mist remains 5 to 7 cm, which is currently met by all formulations. Evenly distributing the product across the entire face, allowing active ingredients to work more effectively, and minimizing product waste all depend on optimal spray dispersion.

Bacterial Identification (Gram Staining)

The purpose of Gram staining is to identify and distinguish microbes. The two main categories of bacteria identified using this approach are Gram-positive and Gram-negative (Jayadi, 2022). The spherical or coccus shape and purple color of Propionibacterium acnes bacteria, as shown in Figure 4.2, indicate that these bacteria are Gram-positive. This is because bacteria can adhere to the first stain, crystal violet, which produces a purple color. Bacteria with a thicker peptidoglycan layer in their cell walls are known as Gram-positive, while those with a thinner layer are known as Gram-negative (Jayadi, 2022).

Antibacterial Activity Testing

Activity testing of red betel leaf extract (Piper crocatum Ruiz & Pav) face mist preparations using Propionibacterium acnes bacteria. This study used concentrations of 15%, 25%, and 35% with a negative control of face mist and a positive control of clindamycin. The mechanism of action of clindamycin involves reversible binding to the 50S ribosomal subunit, which inhibits peptide bond formation and thereby disrupts protein synthesis in bacteria. Additionally, the bacteriostatic or bactericidal properties of clindamycin depend on the concentration of the drug used (Rahmaningtyas, 2022). This study employed the disk diffusion technique using NA medium. Antibacterial activity was assessed by observing whether samples grown on the medium incubated at 37°C for 24 hours showed signs of microbial growth inhibition. This was determined by measuring the clear zone formed around the disk. To determine the inhibitory effect precisely. Antibacterial activity is categorized into four groups: weak, moderate, strong, and very strong. When the diameter of the inhibition zone is less than 5 mm, the antibacterial activity is considered poor. Moderate activity is observed between 5-10 mm, strong activity between 10-20 mm, and very strong activity if greater than 20 mm (Iwani *et al.*, 2024).

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Based on the results of the antibacterial activity test conducted, it was found that all three formulations with concentrations of 15%, 25%, and 35% exhibited antibacterial activity against Propionibacterium acnes, as indicated by the formation of clear zones around the paper discs. The results of each concentration vary. For formulation 1 with a concentration of red betel leaf extract (Piper crocatum Ruiz & Pav.) of 15%, the formulation exhibited moderate antibacterial activity with an average inhibition zone of 7.57 mm. For formulation 2 with a concentration of red betel leaf extract (Piper crocatum Ruiz & Pav.) of 25%, the formulation exhibited strong antibacterial activity with an average inhibition zone of 10.67 mm. For formulation 3 with a concentration of red betel leaf extract (Piper crocatum Ruiz & Pav.) of 35%, the formulation exhibited strong antibacterial activity with an average inhibition zone of 13.47 mm. The positive control used in this study was clindamycin, which exhibited very strong antibacterial activity with an average inhibition zone of 37.6 mm. In the negative control using the face mist base without extract, no inhibition zone was observed. A correlation was observed between the inhibitory activity of the extract studied and the concentration in a previous study on antibacterial activity (Iwani *et al.,* 2024). By varying the concentration, the inhibitory activity was measured at 8.51 mm at 10%, 10.53 mm at 30%, 11.53 mm at 50%, 16.4 mm at 70%, and 20.66 mm at 90%. With an inhibitory effect of 29.15 mm, clindamycin acted as a positive control.

In this study, the concentration of red betel leaf ethanol extract was found to be directly proportional to the size of the inhibition zone formed. The ability of red betel leaf extract to inhibit bacterial growth is due to the presence of active antibacterial compounds such as flavonoids, tannins, alkaloids, and saponins in the plant. Flavonoids are polar compounds that are readily soluble in polar solvents such as ethanol, methanol, butanol, and acetone. As the largest group of phenolic compounds, flavonoids have effective properties in inhibiting the growth of viruses, bacteria, and fungi. Flavonoids and their derivatives play a physiological role as chemical compounds that help plants fight disease through their antibacterial and antiviral properties. The mechanism of action of flavonoids includes damage to bacterial cell membranes, particularly in the phospholipid region, thereby reducing membrane permeability and causing damage to the bacteria (Iwani *et al.,* 2024).

The ability of tannin compounds to form hydrogen bonds with proteins allows them to function as antibacterial agents. Proteins denature when tannins and water form hydrogen bonds, disrupting bacterial metabolism. Another group of chemical compounds, known as alkaloids, has antibacterial effects. Their mechanism involves disrupting the formation of peptidoglycan components in bacterial cell walls, leading to bacterial death. The antibacterial effect of saponin compounds is due to their ability to denature proteins. Saponins can break down bacterial cell membranes by reducing their surface tension and damaging their permeability, similar to the active characteristics of detergents on saponin surfaces (Indratmoko *et al.,* 2023).

The research data were analyzed using SPSS 26 with an initial normality test using the Shapiro-Wilk test, as the sample size was less than 50. The test results showed that all data had a p-value > 0.05, indicating that the data were normally distributed. Homogeneity was tested using the Levene Statistic, yielding a p-value of 0.548, indicating that data from the treatment groups were homogeneous. Since the data were normal and homogeneous, analysis proceeded with an ANOVA test, which yielded a p-value of 0.000 (< 0.05), indicating significant differences in antibacterial activity among the extract concentration groups and the positive control.

The analysis was continued with a Post Hoc test using the LSD method to determine the differences between groups. The results showed that all treatments (F1, F2, F3) exhibited significant differences from each other and the positive control, with a significance level of $p < 0.05$. Formulation

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F1 exhibited significantly lower antibacterial activity compared to F2 (25%) and F3 (35%), with differences of -3.100 (Sig. 0.007) and -5.900 (Sig. 0.000), respectively. This indicates that the 15% extract concentration is still not optimal for achieving maximum antibacterial effects. Compared to the positive control, F1 showed the most significant difference (-30.100, p = 0.000), indicating that the antibacterial inhibitory activity of this formulation is significantly lower than that of the standard antibacterial agent. Thus, a 15% concentration of red betel leaf extract is not yet sufficient to provide optimal antimicrobial protection.

Formulation F2 showed better antibacterial activity compared to F1, as indicated by a positive difference value of +3.100 (Sig. 0.007). However, when compared to F3, F2 still exhibited lower activity (-2.800, p = 0.012). This suggests that increasing the extract concentration from 15% to 25% has a positive impact on antibacterial efficacy, but has not yet achieved its maximum effect. Compared to the positive control, F2 also shows a significant difference (-27,000, Sig. 0.000), indicating that this formulation still requires improvement to match the standard antibacterial efficacy.

Formulation F3 is the formulation with the highest extract concentration and exhibits the highest antibacterial activity among all tested formulations. This is evident from the Post Hoc test results, which show that F3 is significantly better than F1 and F2, with values of +5,900 (p < 0.000) and +2,800 (p = 0.012), respectively. However, when compared to the positive control, F3 still showed a significant difference (-24.200, Sig. 0.000), indicating that although its antibacterial efficacy has improved, this formulation still cannot match the reference antibacterial agent. Thus, the 35% concentration is the most optimal among the three formulations, but it still has limitations compared to the positive control.

The positive control exhibited the highest overall antibacterial activity. All test formulations (F1, F2, and F3) showed significant differences from the positive control, indicating that the active ingredient in the control has a more substantial inhibitory effect on the target bacteria compared to red betel leaf extract at various concentrations. This reinforces that red betel leaf extract does indeed have antibacterial activity, but its effectiveness is still below the antibacterial standard used as a reference.

Conclusion

The results of the study indicate that all face mist formulations containing red betel leaf ethanol extract (Piper crocatum Ruiz & Pav.) meet the physical test parameters, including organoleptic test, homogeneity, pH, and spreadability. The antibacterial activity test against Propionibacterium acnes showed that increasing the extract concentration resulted in greater inhibitory effects, with inhibition zones of 7.57 mm (moderate) at a 15% concentration, 10.67 mm (strong) at a 25% concentration, and 13.47 mm (strong) at a 35% concentration.

The positive control (clindamycin 300 mg) yielded an inhibition zone of 37.60 mm (very strong), while the negative control showed no antibacterial activity. These findings imply that red betel leaf extract has potential as a natural active ingredient in skin care formulations, particularly for the prevention and treatment of acne caused by P. acnes. Higher concentrations may enhance antibacterial efficacy, although they remain below the efficacy level of clindamycin. This study also contributes to the development of safer, environmentally friendly, and natural-based cosmetics that align with the trend toward herbal product use in the cosmetics industry.

The limitations of this study lie in the use of in vitro methods, which cannot fully replicate the efficacy under direct human skin conditions. Additionally, the study only tested one type of acne-causing bacteria and did not evaluate the long-term stability of the formulation. Further research is

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recommended to conduct in vivo tests on human subjects, test efficacy against various acne-causing bacteria, and optimize the formulation to enhance absorption, user comfort, and product stability over extended storage periods.

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