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Antibacterial Activity of Ethanol Extract of Nettle Leaves against *Cutibacterium acnes* and *Pseudomonas aeruginosa*

Zuhairiah Nasution*, Fira Yunita, Murniaty Simorangkir, Saronom Silaban, Nora Susanti

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan, Medan 20221, Indonesia

**Corresponding author : zuhairiahnasution@gmail.com*

ABSTRACT

Acne vulgaris is a common skin disorder associated with the growth of Cutibacterium acnes and Pseudomonas aeruginosa. This study aimed to evaluate the antibacterial activity of ethanol extract of nettle leaves (Urtica dioica) against both bacteria. The extract was obtained by maceration using 96% ethanol and subjected to phytochemical screening. Antibacterial activity was tested using the disc diffusion method at concentrations of 200, 400, and 800 mg/mL. The findings demonstrated that the extract *contained alkaloids, phenolics, flavonoids, steroids, and saponins. The inhibition zones against Cutibacterium acnes were 14.17±0.21 mm, 16.03±0.76 mm, and 17.37±1.33 mm, while against Pseudomonas aeruginosa they were 9.63±0.51 mm, 12.23±0.40 mm, and 14.33±0.47 mm, respectively. Statistical analysis showed significant differences among treatments ($p<0.05$).* These findings indicated that *Urtica dioica leaf extract* may serve as a potential natural antibacterial agent against acne-associated bacteria.

Keywords: *Urtica dioica*, antibacterial activity, *Cutibacterium acnes*, *Pseudomonas aeruginosa*, phytochemical screening.

1. INTRODUCTION

Acne vulgaris is one of the most common chronic skin diseases experienced by adolescents and young adults worldwide. This condition not only affects skin health but also impacts the quality of life of sufferers, including lowering self-confidence, disrupting social interactions, and increasing the risk of psychological disorders. Data from the Global Burden of Disease (GBD) 2021 showed that the prevalence of *acne vulgaris*

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in the 10–24 years age group increased from 8,563 per 100,000 population in 1990 to 9,790 per 100,000 population in 2021, with the highest increase occurring among adolescent females.[1]

The pathogenesis of *acne vulgaris* is multifactorial and involves the role of skin microorganisms. *Cutibacterium acnes* is the main bacterium involved in the development of inflammation through the production of lipase enzymes that break down sebum into free fatty acids, thereby triggering an inflammatory response. Since 2016, *Propionibacterium acnes* has officially been reclassified as *Cutibacterium acnes* based on phylogenetic and genomic studies showing significant differences from other members of the *Propionibacterium* genus.[2,3] In addition to *Cutibacterium acnes*, acne complications may also be aggravated by secondary infections caused by opportunistic bacteria such as *Pseudomonas aeruginosa*, a Gram-negative bacterium capable of forming complex biofilms, thereby increasing its virulence and resistance to antibiotics.[4]

The use of antibiotics, both systemic and topical, remains the primary choice in *acne vulgaris* therapy. Tetracycline-class antibiotics act bacteriostatically by inhibiting protein synthesis through reversible binding to the 30S ribosomal subunit.[5] Several studies have reported that tetracycline is effective against *Cutibacterium acnes*, with inhibition zone diameters ranging from 28–48 mm.[6,7] However, long-term antibiotic use may lead to resistance problems and disrupt the balance of the skin microbiome. It has been reported that approximately 37% of clinical isolates of *Cutibacterium acnes* have developed resistance to two or more antibiotics.[8] Furthermore, prolonged antibiotic therapy may also trigger colonization by Gram-negative bacteria, including *Pseudomonas aeruginosa*, which can cause *pseudomonas folliculitis* or acneiform lesions.[9] These conditions indicate the need for the development of safer and more sustainable antibacterial alternatives.

One approach that has recently gained considerable attention is the utilization of natural ingredients with antibacterial activity. Nettle leaves (*Urtica dioica*) are known to contain flavonoids, saponins, and phenolic compounds with antimicrobial potential.[10] Purwanda reported that cream formulations containing nettle leaf extract at concentrations of 5%, 10%, and 15% were able to inhibit the growth of *Cutibacterium acnes*, with the highest antibacterial activity demonstrated by the 15% concentration, producing an inhibition zone diameter of 16.3 mm and categorized as having strong inhibitory activity.[11] In addition, other studies have shown that nettle leaf extract exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria, with MIC and MBC values of 6.25 mg/mL and 50 mg/mL against *Staphylococcus aureus*, respectively, and bacteriostatic activity against *Escherichia coli* and *Pseudomonas aeruginosa*.[12]

The antibacterial activity of nettle leaves is thought to be associated with the presence of secondary metabolites. Flavonoids are known to damage bacterial cell membranes and inhibit nucleic acid synthesis, while saponins can increase membrane permeability, leading to leakage of bacterial cell contents. Phenolic

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compounds are also capable of denaturing bacterial cell proteins, thereby inhibiting microbial growth. Through these various mechanisms, ethanol extract of nettle leaves has the potential to be developed as a natural antibacterial agent for acne-causing bacteria.

Based on the description above, this study aims to determine the antibacterial activity of ethanol extract of nettle leaves (*Urtica dioica*) against the growth of *Cutibacterium acnes* and *Pseudomonas aeruginosa*. This study is expected to provide scientific information regarding the potential of nettle leaves as a natural antibacterial source that can be further developed for the treatment of skin infections, particularly acne.

2. EXPERIMENTAL

2.1. Chemicals, Equipment and Instrumentation

Fresh nettle leaves (*Urtica dioica*) used in this study were collected from Titi Payung Village, Batu Bara Regency, North Sumatra, Indonesia. The chemicals used included ethanol (C_2H_5OH) 96%, distilled water (H_2O), dimethyl sulfoxide (DMSO; C_2H_6OS) 100%, physiological sodium chloride (NaCl) 0.9%, magnesium powder (Mg), Dragendorff reagent, Mayer reagent, ferric chloride ($FeCl_3$) solution 1%, sulfuric acid (H_2SO_4), glacial acetic acid (CH_3COOH), hydrochloric acid (HCl), sodium chloride solution, gelatin solution, sodium hydroxide (NaOH), sterile paper discs, 0.5 McFarland standard, Nutrient Agar (NA), Mueller Hinton Agar (MHA), and tetracycline. Bacterial cultures of *Cutibacterium acnes* and *Pseudomonas aeruginosa* were obtained from the Microbiology Laboratory of the Postgraduate School, Universitas Sumatera Utara.

The equipment used in this study included a hot plate and magnetic stirrer (IKA), blender (Philips), 60-mesh sieve, Buchner funnel, rotary evaporator, analytical balance (Ohaus), drying oven (Memmert), autoclave, laminar air flow cabinet, incubator, micropipette, vortex mixer, and digital caliper. Glassware used included Erlenmeyer flasks, beakers, measuring cylinders, volumetric flasks, test tubes, Petri dishes, and glass funnels.

2.2. Research Procedure

2.2.1. Preparation of Nettle Leaf (Urtica dioica) Samples

A total of 4 kg of fresh nettle leaves (*Urtica dioica*) were collected and subjected to wet sorting. The sorted leaves were then washed under running water. After wet sorting, the nettle leaves were cut into small pieces and subsequently dried. The dried leaves were ground into powder using a blender, sieved through a 60-mesh sieve, and stored in a container.[13]

2.2.2. Extraction of Nettle Leaf (Urtica dioica) Samples

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A total of 100 g of dried nettle leaf simplicia powder was weighed, and 1,000 mL of 96% ethanol was prepared using a 1:10 (w/v) ratio. The powder was placed into a dark glass container, soaked with ethanol until completely submerged, tightly sealed, stored at room temperature protected from light, and occasionally stirred for 3 × 24 hours. The mixture was then filtered using a Buchner funnel with Whatman No.1 filter paper to obtain the filtrate. The filtrate was subsequently evaporated using a rotary evaporator at approximately 40°C to obtain a concentrated extract.[14]

2.2.3. Phytochemical Screening of Nettle Leaves (Urtica dioica)

Phytochemical screening was carried out to identify the active compounds contained in nettle leaves (*Urtica dioica*). The following tests were conducted on the extract to determine the presence of these compounds.

a). Flavonoid Test

A total of 40 mg of concentrated extract was mixed with 100 mL of hot water, heated for 5 minutes, and filtered. Five milliliters of the filtrate were then added with 50 mg of magnesium powder and 1 mL of concentrated HCl, followed by vigorous shaking. A yellow, orange, or red coloration indicated the presence of flavonoids.[15]

b). Alkaloid Test

A total of 40 mg of concentrated extract was added to 2 mL of chloroform and 2–3 drops of ammonia, then filtered. The obtained filtrate was subsequently mixed with 3–5 drops of concentrated sulfuric acid (H_2SO_4) and shaken until two layers were formed. The lower acidic layer was collected and added with 4–5 drops each of Mayer and Dragendorff reagents. The formation of a white precipitate with Mayer reagent and a reddish-yellow precipitate with Dragendorff reagent indicated the presence of alkaloids.[15]

c). Triterpenoid and Steroid Test

A total of 40 mg of concentrated extract was mixed with 10 drops of glacial acetic acid (CH_3COOH) and 2 drops of concentrated H_2SO_4. The extract was considered positive for steroids if the solution turned green or blue, whereas a red or purple color indicated the presence of triterpenoids.[15]

d). Saponin Test

A total of 40 mg of concentrated extract was added to 10 mL of water and shaken for 1 minute. Afterward, 2 drops of 1 N HCl were added. The sample was considered positive for saponins if stable foam persisted for approximately 7 minutes.[15]

e). Tannin Test

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A total of 40 mg of concentrated extract was mixed with 1 mL of NaCl solution (20 mg/mL) and 5 mL of gelatin solution (10 mg/mL). The formation of a precipitate indicated the presence of tannins.[15]

f). Phenolic Test

A total of 40 mg of concentrated extract was added with 10 drops of $FeCl_3$ solution (10 mg/mL). The sample was considered positive for phenolic compounds if the solution changed color to green, blue, purple, red, or dark black.[15]

2.2.4. Preparation and Sterilization of Equipment

All equipment used in this study was first cleaned and dried. Glassware such as test tubes, Erlenmeyer flasks, measuring cylinders, vials, and pipettes were covered with cotton wrapped in gauze and then wrapped with parchment paper before being sterilized in an autoclave at 121°C for 15 minutes.

Metal instruments such as forceps and inoculating loops were sterilized by flaming over a Bunsen burner for several seconds until red hot. The Laminar Air Flow (LAF) workspace was cleaned of dust, sprayed with 70% ethanol, and left for approximately 15 minutes to maintain sterility before use. All procedures were carried out aseptically to prevent contamination during antibacterial testing.[16]

2.2.5. Preparation of Media

In this study, Nutrient Agar (NA) medium was used for bacterial rejuvenation. The medium was prepared by dissolving 2.8 g of NA powder in 100 mL of distilled water in an Erlenmeyer flask. The mixture was homogenized by heating in a water bath until boiling. After homogenization, the medium was sterilized in an autoclave at 121°C for 15 minutes. The sterilized medium was then cooled to an appropriate temperature before use, while unused medium was stored in a refrigerator.

Mueller Hinton Agar (MHA) medium was used for antibacterial activity testing. The medium was prepared by dissolving 3.8 g of MHA powder in 100 mL of distilled water in an Erlenmeyer flask. The mixture was stirred until homogeneous and heated until completely dissolved and boiling. The medium was then sterilized in an autoclave at 121°C for 20 minutes. Approximately 20 mL of sterile medium was poured aseptically into Petri dishes and incubated at 37°C before use in antibacterial testing.[17]

2.2.6. Bacterial Rejuvenation

One colony each of *Cutibacterium acnes and Pseudomonas aeruginosa* was taken using a sterile inoculating loop and inoculated onto the surface of slanted Nutrient Agar medium using the streak plate method. The cultures were then incubated at 37°C for 24 h to obtain active bacterial cultures.[16]

2.2.7. Preparation of Bacterial Suspension (0.5 McFarland)

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Twenty-four-hour-old bacterial cultures were taken from slanted Nutrient Agar medium using two loops of bacterial colonies and transferred into test tubes containing 5 mL of 0.9% NaCl solution. The suspension was homogenized using a vortex mixer. The turbidity of the suspension was adjusted to match the 0.5 McFarland standard, equivalent to approximately 10^8 CFU/mL bacterial concentration.[16]

2.2.8. Preparation of Stock Solution and Concentration Series of Ethanol Extract of Nettle Leaves (Urtica dioica)

The stock solution of ethanol extract of nettle leaves (*Urtica dioica*) was prepared by dissolving 8 g of concentrated extract in 100% DMSO to a final volume of 10 mL, resulting in a concentration of 800 mg/mL. From this stock solution, concentration series of 200 mg/mL, 400 mg/mL, and 800 mg/mL were prepared.

The 200 mg/mL concentration was prepared by taking 125 µL of the stock solution and adding DMSO until the final volume reached 500 µL. The 400 mg/mL concentration was prepared by taking 250 µL of the stock solution and adding DMSO to a final volume of 500 µL. Meanwhile, the 800 mg/mL concentration was obtained directly from 500 µL of the stock solution without additional dilution.[15]

2.2.9. Antibacterial Activity Test of Ethanol Extract of Nettle Leaves (Urtica dioica)

Antibacterial activity was evaluated using the disc diffusion method. A total of 200 µL of *Cutibacterium acnes* and *Pseudomonas aeruginosa* suspensions were inoculated onto Mueller Hinton (MH) agar media and evenly spread using a spreader. Blank discs were then placed on the media surface and treated with 20 µL of each treatment consisting of 100% DMSO as the negative control, ethanol extract of nettle leaves (*Urtica dioica*) at concentrations of 200 mg/mL, 400 mg/mL, and 800 mg/mL, and tetracycline 20 mg/mL as the positive control. The media were incubated at 37°C for 18–24 hours, after which the inhibition zone diameters around each disc were measured. Each treatment was performed in triplicate.[15]

2.2.10. Data Analysis

The obtained data were analyzed using IBM SPSS Statistics 22 software. Data normality was tested using the Shapiro–Wilk test, followed by Levene's homogeneity test. Data that were normally distributed and homogeneous were further analyzed using One Way ANOVA. If a significance value of $p<0.05$ was obtained, the analysis was continued with the Tukey HSD test to determine significantly different treatment groups.[18]

3. RESULTS AND DISCUSSION

3.1. Preparation of Nettle Leaves (Urtica dioica)

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Fresh nettle leaves (Urtica dioica) were used as the plant material in this study. (*Urtica dioica*) characterized by green color, non-wilted appearance, absence of holes, and good physical condition. The samples were collected from Titi Payung Village, Batu Bara Regency, North Sumatra, Indonesia.

Preparation of nettle leaves was conducted to obtain simplicia suitable for the extraction process. Fresh leaves were sorted and washed under running water to remove adhering impurities, followed by drying to reduce the moisture content. The dried samples were then ground into powder and sieved using a 60-mesh sieve to obtain simplicia powder with uniform particle size.

3.2. Extraction of Nettle Leaves (Urtica dioica)

Dried nettle leaf simplicia was ground into powder using a blender. The simplicia powder was subsequently macerated using 96% ethanol for 3 × 24 h with occasional stirring. After the maceration process was completed, the filtrate was separated from the residue through filtration. The filtrate was then concentrated using a rotary evaporator at 50°C to obtain a viscous extract of nettle leaves.

The extract yield was obtained from the concentration process of ethanol extract of nettle leaves (*Urtica dioica*). The resulting viscous extract was weighed to determine its final weight, followed by calculation of the extract yield using the following formula. The yield of ethanol extract of nettle leaves (*Urtica dioica*) was presented in Table 1.

$$\text{Yield } (\%) = \frac{\text{Extract Weight}}{\text{Simplicia Weight}} \times 100$$

Table 1. Yield of Ethanol Extract of Nettle Leaves (*Urtica dioica*)

No	Simplicia Weight	Viscous Extract Weight	Yield	Color
1.	100 g	12.5 g	12.5 %	Dark green

The extraction yield of *Urtica dioica* ethanol extract obtained in this study was 12.5%. Yield is defined as the comparison between the weight of the extract obtained and the initial weight of the simplicia after the extraction and solvent evaporation processes.[19] The yield value indicates that 96% ethanol was able to extract secondary metabolite compounds contained in nettle leaves effectively.

Maceration was selected as the extraction method in this study. This method was selected because it is relatively simple, easy to perform, and widely used in the extraction of natural materials. During the maceration process, the solvent penetrates the cell walls of the simplicia and dissolves the active compounds present inside the cells. Differences in concentration between the liquid inside and outside the cells cause the active compounds to diffuse into the solvent until equilibrium is achieved.[19]

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The selection of 96% ethanol as the extraction solvent was based on its ability to dissolve polar and semi-polar secondary metabolites such as flavonoids, alkaloids, tannins, and saponins. In addition, ethanol is considered effective for extracting plant compounds with antibacterial potential. Ethanol is one of the most commonly used solvents in the extraction of *Urtica dioica* for antibacterial activity testing against Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[20]

The obtained extract appeared as a thick dark-green viscous mass. This coloration was likely caused by chlorophyll pigments dissolved during the extraction process using ethanol.[21] The yield value can be influenced by several factors, including the type of solvent, extraction method, extraction temperature and duration, particle size of the simplicia, and the preprocessing treatment of the raw materials before extraction.[19]

3.3. Phytochemical Screening of Nettle Leaves (Urtica dioica)

The results of qualitative phytochemical screening of ethanol extract of nettle leaves (*Urtica dioica*) are presented in Table 2.

Table 2. Phytochemical Screening Results of Nettle Leaves (*Urtica dioica*)

No.	Test	Reagent	Result	Observation
1	Alkaloid Test	Mayer reagent	+	White precipitate
		Dragendorff reagent	+	Orange precipitate
2	Phenolic Test	$FeCl_3$ 1%	+	Green-colored solution
3	Flavonoid Test	Mg powder + concentrated HCl	+	Yellow-colored solution
4	Steroid Test	CH_3COOH + concentrated H_2SO_4	+	Green-colored solution
5	Triterpenoid Test	CH_3COOH + concentrated H_2SO_4	–	No red or purple coloration
6	Saponin Test	Distilled water + HCl 1 N	+	Stable foam for ± 7 min
7	Tannin Test	NaCl + gelatin	–	No precipitate formed

The phytochemical screening results showed that the ethanol extract of *Urtica dioica* contained several secondary metabolites, namely alkaloids, phenolics, flavonoids, steroids, and saponins. Meanwhile, triterpenoid and tannin tests showed negative results. The presence of these secondary metabolites indicates that nettle leaves have potential to be utilized as natural materials in the pharmaceutical field, particularly as antibacterial agents.[22]

Alkaloid testing using Mayer reagent produced a white precipitate, while Dragendorff reagent formed an orange precipitate. These results indicated the presence of alkaloid compounds in the ethanol extract of nettle leaves. The white precipitate formed with Mayer reagent was caused by the interaction between nitrogen atoms in alkaloids and potassium tetraiodomercurate ions, forming an insoluble complex. Meanwhile, the orange precipitate formed with Dragendorff reagent resulted from the formation of potassium alkaloid-bismuth iodide complexes.[23]

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In the phenolic test, the addition of 1% $FeCl_3$ reagent produced a green color change in the solution, indicating the presence of phenolic compounds in the extract. This color change occurred due to the reaction between Fe^{3+} ions and hydroxyl groups in phenolic compounds, resulting in the formation of colored complexes.[24]

Flavonoid testing using magnesium powder and concentrated HCl produced a yellow coloration in the solution. This result indicated the presence of flavonoid compounds in the ethanol extract of nettle leaves. The color change occurred due to the reduction of the flavonoid benzopyrone nucleus by magnesium metal under acidic conditions, resulting in the characteristic flavonoid color.[25]

In the steroid test using a mixture of acetic anhydride and concentrated sulfuric acid, the solution changed to green. This result indicated the presence of steroid compounds in the extract. The green coloration occurred due to the Liebermann–Burchard reaction between the steroid nucleus and strong acid reagents.[25] The results of this study were also consistent with previous findings reporting that ethanol extract of nettle leaves (*Urtica dioica* L.) positively contained steroid compounds.[26]

In the triterpenoid test, no red or purple coloration was observed after the addition of reagents, indicating a negative result for triterpenoid compounds. These findings indicated that triterpenoid compounds were either absent or present in concentrations too low to be detected by the qualitative method used, making them undetectable through the qualitative test performed. These results differed from previous studies reporting the presence of triterpenoid compounds in nettle leaves.[22] Such differences may be influenced by several factors, including solvent type, extraction method, plant material conditions, and environmental factors affecting plant growth.

The saponin test showed the formation of stable foam for approximately ±7 minutes after the addition of distilled water and 1 N HCl. Foam formation indicated the presence of saponin compounds, which possess surface-active properties due to their polar and nonpolar groups. A positive saponin result was characterized by foam remaining stable after the addition of 1 N HCl.[27]

In the tannin test using NaCl and gelatin reagents, no precipitate was formed, indicating that the ethanol extract of nettle leaves was negative for tannin compounds. The absence of precipitate suggested that tannin levels in the extract were either very low or undetectable through the qualitative method used. These findings differed from previous studies reporting that nettle leaves (*Urtica dioica* L.) positively contained tannins.[28] Differences in research results may be influenced by solvent type, extraction method, compound concentration in the sample, and environmental conditions where the plants were grown.

3.4. Preparation and Sterilization of Equipment

Equipment sterilization was carried out prior to antibacterial activity testing to maintain sterility and prevent contamination that could affect the research results. Glassware such as test tubes, Erlenmeyer flasks,

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measuring cylinders, vials, and pipettes were sterilized using an autoclave at 121°C for 15 min because this method is capable of eliminating microorganisms, including bacterial spores. Prior to sterilization, the equipment was covered with cotton and wrapped in parchment paper to maintain sterility until use. Metal instruments such as forceps and inoculating loops were sterilized by flaming over a Bunsen burner until red-hot to remove microorganisms attached to the surface of the equipment.

In addition to equipment sterilization, the working area in the Laminar Air Flow (LAF) cabinet was cleaned and sprayed with 70% ethanol to minimize the risk of contamination during the testing process. The use of 70% ethanol as a disinfectant is considered effective because it can denature microbial cell proteins. All procedures were carried out aseptically to maintain sterile conditions, thereby ensuring that the antibacterial activity test results obtained were more accurate and reliable.

3.5. Preparation of Culture Media

Nutrient Agar (NA) medium was used for bacterial rejuvenation prior to antibacterial activity testing. This medium was selected because it contains nutrients capable of supporting bacterial growth effectively. The medium was heated until completely dissolved and homogeneous to ensure uniform mixing of all components. Subsequently, the medium was sterilized using an autoclave at 121°C for 15 min to prevent microbial contamination. The sterilized medium was then cooled before being used for bacterial inoculation.

Mueller Hinton Agar (MHA) medium was used for antibacterial activity testing because it possesses good diffusion properties in the disc diffusion method. The medium was prepared by dissolving MHA powder in distilled water followed by heating until completely dissolved and homogeneous. Sterilization using an autoclave was performed to maintain media sterility prior to use. The sterile medium was then aseptically poured into Petri dishes and incubated at 37°C to support optimal growth of the test bacteria during antibacterial activity testing.

3.6. Rejuvenation of Bacterial Cultures

Bacterial rejuvenation was carried out to obtain active bacterial cultures for antibacterial activity testing. Bacterial isolates were inoculated onto slanted Nutrient Agar (NA) medium using a sterile inoculating loop with the streak plate method. Nutrient Agar medium was selected because it provides sufficient nutrients to support the optimal growth of *Cutibacterium acnes* and *Pseudomonas aeruginosa*.

The inoculated plates were incubated at 37°C for 18–24 h. at 37°C for 24 h to allow bacterial growth and obtain active cultures. During the rejuvenation process, all procedures were performed aseptically to prevent contamination by other microorganisms that could affect the research results. Bacterial growth on slanted agar medium was indicated by the formation of a white layer on the surface of the medium, as shown in Figure 1.

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Figure 1. Rejuvenated Cultures of *Cutibacterium acnes* and *Pseudomonas aeruginosa*

3.7. Preparation of Bacterial Suspension (0.5 McFarland)

The preparation of bacterial suspensions was carried out to obtain a uniform number of bacteria before antibacterial activity testing. Twenty-four-hour-old bacterial cultures were used because they were in the active growth phase, making them more suitable for antibacterial testing. The bacterial suspensions were prepared using 0.9% NaCl solution, which is isotonic and capable of maintaining the stability and integrity of bacterial cells during the dilution process. Furthermore, homogenization was performed using a vortex mixer to ensure that the bacterial suspension was evenly mixed.

The turbidity of the bacterial suspension was adjusted according to the 0.5 McFarland standard to obtain a uniform bacterial concentration in each treatment. This standard was used as a reference for estimating the number of bacterial cells in the suspension so that the antibacterial activity test results would be more consistent and comparable among treatments. The turbidity level of the 0.5 McFarland standard is equivalent to a bacterial concentration of approximately 1.5×10^8 CFU/mL.[29]

3.8. Antibacterial Activity Test of Ethanol Extract of Nettle Leaves (Urtica dioica)

The antibacterial activity test of ethanol extract of nettle leaves was carried out to determine the ability of the extract to inhibit the growth of *Cutibacterium acnes* and *Pseudomonas aeruginosa*. The disc diffusion method was selected because it is capable of demonstrating antibacterial activity through the formation of clear inhibition zones surrounding the test discs. In this study, extract concentrations of 200 mg/mL, 400 mg/mL, and 800 mg/mL were used to evaluate the effect of concentration increase on bacterial growth inhibition.

Tetracycline 20 mg/mL was used as a positive control because it is known to possess strong antibacterial activity, whereas 100% DMSO was used as a negative control to ensure that the solvent did not influence the test results. The inoculated plates were incubated at 37°C for 18–24 h. Antibacterial activity was evaluated based on the formation of inhibition zones around the paper discs, where larger inhibition zone diameters indicated stronger antibacterial activity against the test bacteria. The measurement results of inhibition zone diameters of ethanol extract of nettle leaves (*Urtica dioica*) are presented in Table 3

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Table 3. Antibacterial Activity of *Urtica dioica* Ethanolic Extract Against *Cutibacterium acnes* and *Pseudomonas aeruginosa* Based on Inhibition Zone Diameter

No	Test Bacteria	Test Concentration (mg/mL)	Inhibition Zone (mm) R1	R2	R3	Mean Inhibition Zone	Inhibitory Response
1.	*Cutibacterium acnes*	200	14,10	14,40	14,00	14,17±0,21	Strong
		400	15,50	16,90	15,70	16,03±0,76	Strong
		800	16,50	18,90	16,70	17,37±1,33	Strong
		k^+	28,40	27,20	25,90	27,17±1,25	Very Strong
2.	*Pseudomonas aeruginosa*	200	9,50	10,20	9,20	9,63±0,51	Moderate
		400	12,30	11,80	12,60	12,23±0,40	Strong
		800	14,50	13,80	14,70	14,33±0,47	Strong
		k^+	25,20	24,80	25,90	25,30±0,56	Very Strong

As shown in Table 3, the inhibition zone diameter increased with increasing extract concentration for both tested bacteria. Representative images of the antibacterial activity observed during the disc diffusion assay are presented in Figure 2.

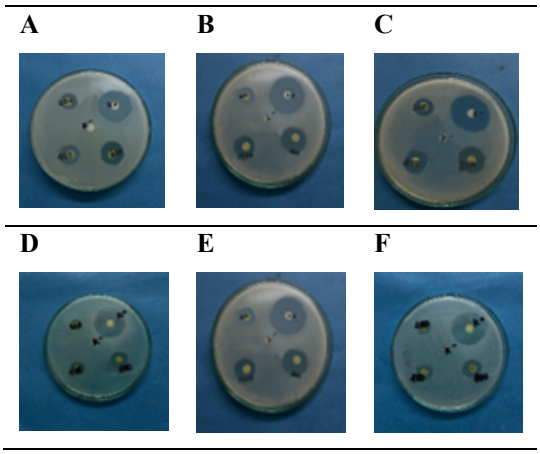

Figure 2. Representative inhibition zones produced by *Urtica dioica* ethanolic extract against *Cutibacterium acnes* (A–C) and *Pseudomonas aeruginosa* (D–F) as determined by the disc diffusion method.

Based on the research results, the ethanol extract of nettle leaves demonstrated the ability to inhibit the growth of both tested bacteria at all concentration variations. In *Cutibacterium acnes*, the average inhibition zone diameter produced at a concentration of 200 mg/mL was 14.17±0.21 mm, which increased to 16.03±0.76 mm at 400 mg/mL and 17.37±1.33 mm at 800 mg/mL. All of these results were classified as strong antibacterial activity according to the antibacterial activity classification, where inhibition zone diameters of 10–20 mm are categorized as strong.[30] The positive control produced an average inhibition zone of 27.17±1.25 mm, which was classified as very strong, while the negative control showed no inhibition zone formation.

In the antibacterial test against *Pseudomonas aeruginosa*, the concentration of 200 mg/mL produced an average inhibition zone diameter of 9.63±0.51 mm, which was categorized as moderate. Increasing the extract concentration to 400 mg/mL and 800 mg/mL increased the inhibition zone diameters to 12.23±0.40 mm and 14.33±0.47 mm, respectively, both of which were categorized as strong. Meanwhile, the positive

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control produced an average inhibition zone diameter of 25.30±0.56 mm, which was classified as very strong, whereas DMSO as the negative control did not exhibit any inhibitory activity against the tested bacteria.

Data analysis in this study was performed using IBM SPSS Statistics 22 software with a 95% confidence level to evaluate the effect of ethanol extract of nettle leaves on antibacterial activity against *Cutibacterium acnes* and *Pseudomonas aeruginosa*. The mean inhibition zone diameter data were initially analyzed using the Shapiro–Wilk normality test because the number of samples was less than 100. In the Shapiro–Wilk test, a significance value of $p > 0.05$ indicated that the data were normally distributed, whereas $p < 0.05$ indicated non-normal distribution.

Subsequently, homogeneity testing was conducted using Levene's test to evaluate the equality of variances among treatment groups. Data that fulfilled the assumptions of normality and homogeneity were further analyzed using One Way ANOVA to determine significant differences among treatment groups. When the One Way ANOVA test showed a significance value of $p < 0.05$, the analysis was continued using the Tukey Post Hoc test to identify treatment groups with significant differences. The statistical analysis results were presented in Table 4.

Table 4. SPSS Analysis Results of Treatment Groups against *Cutibacterium acnes* and *Pseudomonas aeruginosa*

Test Bacteria	Treatment	Shapiro–Wilk Normality	Levene Homogeneity	One Way ANOVA
Cutibacterium acnes	E200	0.463		
	E400	0.253	0.930	0.000
	E800	0.144		
	Positive control	0.956		
Pseudomonas aeruginosa	E200	0.567		
	E400	0.726	0.930	0.000
	E800	0.407		
	Positive control	0.702		

Source: Processed data using IBM SPSS Statistics 22 (2026).

Based on the Shapiro–Wilk normality test results, the significance values for *Cutibacterium acnes* were 0.463 for E200, 0.253 for E400, 0.144 for E800, and 0.956 for the positive control (K+). Meanwhile, for *Pseudomonas aeruginosa*, the significance values obtained were 0.567 for E200, 0.726 for E400, 0.407 for E800, and 0.702 for the positive control (K+). These results indicate that all treatment groups had significance values of $p > 0.05$, demonstrating that the data were normally distributed.

The analysis was subsequently followed by Levene's homogeneity test. The homogeneity test results for both test bacteria showed a significance value of 0.930, indicating that the data were homogeneous because the significance value was greater than 0.05.

One Way ANOVA analysis was then performed to evaluate differences in the mean inhibition zone diameters among treatment groups. The analysis results for both bacteria showed a significance value of

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0.000, indicating statistically significant differences among treatment groups because $p < 0.05$. Therefore, the analysis was continued using the Tukey Post Hoc test to identify treatment groups with significant differences.

Table 5. Tukey Post Hoc Test Results against *Cutibacterium acnes* and *Pseudomonas aeruginosa*

Test Bacteria	Group (I)	Group (J)	Significance	Interpretation
Cutibacterium acnes	E200	E400	0.001	Significantly different
		E800	0.000	Significantly different
		K+	0.000	Significantly different
	E400	E200	0.001	Significantly different
		E800	0.003	Significantly different
		K+	0.000	Significantly different
	E800	E200	0.000	Significantly different
		E400	0.003	Significantly different
		K+	0.000	Significantly different
	K+	E200	0.000	Significantly different
		E400	0.000	Significantly different
		E800	0.000	Significantly different
Pseudomonas aeruginosa	E200	E400	0.001	Significantly different
		E800	0.000	Significantly different
		K+	0.000	Significantly different
	E400	E200	0.001	Significantly different
		E800	0.003	Significantly different
		K+	0.000	Significantly different
	E800	E200	0.000	Significantly different
		E400	0.003	Significantly different
		K+	0.000	Significantly different
	K+	E200	0.000	Significantly different
		E400	0.000	Significantly different
		E800	0.000	Significantly different

Source: Processed data using IBM SPSS Statistics 22 (2026).

The Tukey Post Hoc test results demonstrated that all treatment groups against both *Cutibacterium acnes* and *Pseudomonas aeruginosa* showed significant differences with $p < 0.05$. The E200 group differed significantly from E400, E800, and the positive control (K+). Similarly, the E400 group showed significant differences compared to E800 and K+. In addition, the E800 group also differed significantly from the positive control group. These findings indicated that increasing the concentration of ethanol extract of nettle leaves enhanced the antibacterial activity against both test bacteria.

The increase in inhibition zone diameter at each concentration level indicated that extract concentration affected antibacterial activity. Higher extract concentrations contain greater amounts of active compounds that diffuse into the media, thereby enhancing the ability to inhibit bacterial growth. These findings are consistent with previous studies stating that increasing the concentration of antibacterial compounds can enhance inhibitory activity against bacterial growth.[31]

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The higher antibacterial activity of ethanol extract of nettle leaves against *Cutibacterium acnes* compared to *Pseudomonas aeruginosa* may be related to differences in the cell wall structures of the two bacteria. Gram-positive bacteria such as *Cutibacterium acnes* possess a thick peptidoglycan layer but have a simpler cell wall structure, allowing antibacterial compounds to penetrate bacterial cells more easily. In contrast, *Pseudomonas aeruginosa* as a Gram-negative bacterium has an outer membrane composed of lipopolysaccharides and lipoproteins with higher lipid content, resulting in lower permeability to antibacterial compounds.[32]

4. CONCLUSION

Phytochemical screening confirmed that ethanol extract of *Urtica dioica* leaves contained alkaloids, phenolics, flavonoids, steroids, and saponins. The extract exhibited antibacterial activity against *Cutibacterium acnes* and *Pseudomonas aeruginosa* at concentrations of 200, 400, and 800 mg/mL, with the strongest inhibitory effect observed at 800 mg/mL.

The inhibition zone diameters against *Cutibacterium acnes* were 14.17 ± 0.21 mm, 16.03 ± 0.76 mm, and 17.37 ± 1.33 mm, respectively, while against *Pseudomonas aeruginosa* they were 9.63 ± 0.51 mm, 12.23 ± 0.40 mm, and 14.33 ± 0.47 mm. Statistical analysis demonstrated significant differences among extract concentrations ($p < 0.05$), indicating that ethanol extract of *Urtica dioica* leaves possesses potential as a natural antibacterial agent against acne-associated bacteria.

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