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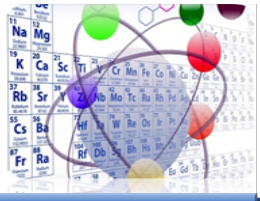

Antibacterial Activity of Blue Pea Flower (*Clitoria ternatea*) Acetone Extract Against *Propionibacterium acnes* and *Citrobacter freundii* Bacteria

Tita Juwitaningsih*, Hidayani

Departement of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Medan, 20221, Indonesia

**Corresponding author : juwitaningsih@unimed.ac.id*

ABSTRACT

This study aims to determine the antibacterial activity of acetone extract of butterfly pea flower (C. ternatea) against P. acne and C. freundii bacteria and to identify the secondary metabolites contained in acetone extract of butterfly pea flower (C. ternatea). Butterfly pea flowers were extracted using the maceration method with acetone solvent. Preliminary antibacterial activity tests were conducted using the disk diffusion method. This was followed by the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method. Phytochemical screening tests were conducted to identify secondary metabolite compounds. The results showed that the MIC and MBC values against P. acne and C. freundii bacteria were 1250 µg/mL and >5000 µg/mL, respectively. The phytochemical screening test showed that the acetone extract of blue pea flower contained flavonoids, saponins, tannins, and steroids.

Keywords : *C. ternatea, Antibacterial, P. acne, C. freundii, Phytochemical Screening.*

1. INTRODUCTION

Infectious diseases are one of the main problems in the field of health. These infectious diseases are caused by pathogenic microorganisms that enter the human body and develop inside it, causing damage to organs [19]. One of the most common infectious diseases is acne. Acne is an infection of the pilosebaceous glands characterized by the appearance of comedones, papules, pustules, and nodules. Acne is caused by the bacterium *Propionibacterium acnes*. *P. acnes* causes inflammation of the skin on the face, characterized by redness around the pimples and pus-filled pimples [11]. Acne can be transmitted through direct contact with sensitive skin, the use of cosmetic tools, and the sharing of skin care products. Acne can be treated based on the clinical

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type of acne and its severity. A commonly used treatment for acne is topical antibiotics, as this type of antibiotic is susceptible to *Propionibacterium acnes* resistance [3].

In addition to acne, another type of infectious disease experienced by the community is urinary tract infection. Urinary tract infections are infections that occur when pathogenic bacteria enter the kidneys, ureters, bladder, or urethra [25]. Urinary tract infections occur due to bacterial inflammation in the urinary tract, which starts in the renal cortex and spreads to the meatus, accompanied by bacterial colonization in the urine. This infection is transmitted through direct sexual contact and contact with infected patients. Urinary tract infections can be treated depending on the severity of the infection and the patient's condition. The general treatment for urinary tract infections is oral antibiotics, such as amoxicillin and ampicillin, which are effective against *Citrobacter freundii* bacteria [13].

Infectious diseases can be treated using herbal plants. One herbal plant that can be used as medicine is the butterfly pea flower. The butterfly pea flower, also known by its scientific name *Clitoria ternatea*, has petals with unique colors, such as purple, blue, pink, and white [6]. The butterfly pea flower (*C. ternatea*) is a plant from the Fabaceae family. This plant is believed to originate from the Indian Ocean or South China Sea region. The butterfly pea flower is blue in color, which comes from the anthocyanin compound [21]. Butterfly pea flowers have many pharmacological potentials, such as antioxidant, antibacterial, anti-inflammatory, analgesic, antiparasitic, antacid, antidiabetic, anticancer, antihistamine, immunomodulator, and role in the central nervous system [15]. Due to its compound content, butterfly pea flowers have been proven to provide many benefits for human life. These benefits are believed to be due to the presence of bioactive components derived from various types of secondary metabolites. Secondary metabolites are organic compounds produced by plants, but do not play a direct role in activities such as photosynthesis, growth, respiration, water absorption, transport, protein synthesis, nutrient absorption, cell differentiation, and the synthesis of carbohydrates, proteins, and fats. These secondary metabolites are usually only found in one type of plant or certain species groups. They differ from primary metabolites such as amino acids, nucleotides, sugars, and fats, which are commonly found in almost all plants [1].

The antibacterial activity of butterfly pea flower extract has been tested against gram-positive and gram-negative bacteria. Previous studies have mostly used ethanol, methanol, and water as solvents. In this study, acetone was used as a solvent to extract butterfly pea flowers, and antibacterial activity testing and phytochemical screening were conducted.

2. EXPERIMENTAL

The main samples used in this study were butterfly pea flowers (*C. ternatea*) obtained from our own cultivation in Deli Serdang Regency, North Sumatra, and redistilled acetone as the solvent. The chemicals used included acetone (Merck), distilled water, *Mueller Hinton Agar* (MHA) (Oxoid CM0337), *Mueller Hinton Broth* (MHB), *P. acne* bacteria, *C. freundii* bacteria, dimethyl sulfoxide (DMSO) (Merck), 0.9% physiological NaCl, chloramphenicol paper (Oxoid), McFarland 0.5%, blank disc paper (Oxoid), Whatman filter paper, aluminum foil, Mayer's reagent, Dragendorff's reagent, Wagner's reagent, concentrated sulfuric acid, ferric chloride, and anhydrous acid.

2.1 Sample Preparation and Extraction

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Sample preparation begins with cleaning the butterfly pea flowers of any dirt and drying them in the open air. After that, the butterfly pea flowers are crushed. The crushed flowers are stored in a sealed container. Extraction is carried out by weighing 450 grams of butterfly pea flower simplisia, placing it in a glass maceration bottle, and adding acetone solvent until the simplisia is submerged. The maceration process is carried out for 3x24 hours. The sample is filtered using a Buchner funnel, then the filtrate is placed in a rotary evaporator until a thick butterfly pea flower extract (*C. ternatea*) is obtained.

2.2 Sample Concentration Preparation

Before conducting antibacterial testing, the solution to be tested is prepared by weighing 100 mg of extract. The extract is then dissolved in 1000 mL of 100% DMSO and stirred using a vortex. Next, 100 µg/mL is taken and diluted in 900 mL of DMSO, resulting in a 1% (10,000 ppm) test solution in 10% DMSO [4].

2.3 Preparation of Bacterial Inoculum

A single colony of bacteria was collected using a cotton swab, then placed in a vial containing 3 mL of 0.9% NaCl. The vial was then vortexed to achieve a turbidity equivalent to that of a 0.5% McFarland Standard solution, which is 1.5 x 107 CFU/mL [29].

2.4 Antibacterial Activity Test

2.4.1 Disk Diffusion Test

The paper disk diffusion test begins by placing 100 µL of bacterial inoculum onto MHA media, then spreading it evenly using an L-shaped spreader and allowing it to sit for a moment until the inoculum dries on the media. The paper disc is placed and pressed gently onto the surface of the MHA medium, then 20 µL of the test solution is dripped onto the surface of the paper disc. The same is done with the DMSO solvent as a negative control. Conversely, a 20 µg chloramphenicol paper disc is placed on the surface of the MHA medium as a positive control. Then the Petri dish is covered and wrapped tightly, then placed in an incubator at a temperature of 37°C for 18/24 hours. After 18/24 hours of incubation, each Petri dish is examined to see the clear zone that has formed. Next, the clear zone is measured using a caliper, so that the diameter of the bacterial growth inhibition zone is obtained [5].

2.4.2 MIC (Minimum Inhibitory Concentration) Test Using the Microdilution Method

The microdilution method begins by adding 1 loopful of bacteria to 3000 µL of 0.9% NaCl solution and then vortexing. A total of 100 µL of this mixture is taken and mixed into 9,900 µL of MHB medium. To maintain cleanliness, a microplate consisting of 96 wells is worked on in a laminar flow. The MIC test is carried out by placing 100 µL of MHB medium into the first column as a negative control. Next, 100 µL of MHB medium containing bacteria (positive control) was added to the second to twelfth columns of the microplate. A 1% concentration of 100 µL test solution was added to the twelfth column, and then 100 µL was taken to create a dilution series. A total of 100 µL of test solution was transferred from column twelve to column eleven, and from column eleven, 100 µL was taken to be added to column ten. This process was continued until the third column. Each column contained 100 µL of solution. After that, the microplate was incubated at 37º C for 24 hours [5].

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The MIC value is determined by comparing the positive control column (column two) with the sterile column (column one), then each column is compared with the positive column. The MIC value is determined based on the total inhibition of bacterial growth in the column using a specific concentration of the test solution. MIC is defined as the lowest concentration of an antimicrobial agent capable of inhibiting all bacterial growth visibly [5].

2.4.3 MBC (Minimum Bacteriocidal Concentration) Test using the Microdilution Method

The MBC test is performed by inoculating all test solutions, namely by taking 10 µL from each of the best wells on the microplate (columns 1-12), then planting them on MHA agar media and incubating at 37°C for 24 hours. MBC is defined as the lowest concentration of the test solution capable of killing bacteria [17].

2.5 Phytochemical Screening Test

The acetone extract of butterfly pea flowers was dissolved in acetone solvent, then a phytochemical screening test was conducted to identify secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and terpenoids [26].

2.5.1 Alkaloids

The presence of flavonoid compounds was tested by dissolving 0.5 grams of the sample in a test tube, then adding 2 mL of 2N HCl and heating. After cooling, the filtrate was filtered and divided into three test tubes. Next, 1 mL of Mayer's reagent, Dragendorf's reagent, and Wagner's reagent are added to each tube. If alkaloids are present in the sample, a white precipitate will form in the test tube containing Mayer's reagent. Meanwhile, a red precipitate will form in the test tubes containing Dragendorf's and Wagner's reagents.

2.5.2 Flavonoids

The presence of flavonoid compounds is tested by placing 5 mL of extract into a test tube, then adding 5 drops of H_2SO_4. The presence of flavonoid compounds in the sample is indicated by a change in the color of the solution to red, yellow, green, or brown.

2.5.3 Saponin

The test for the presence of saponin compounds is carried out by placing 0.5 grams of the sample in a test tube and adding distilled water, then shaking until a stable foam appears for 5 minutes.

2.5.4 Tannin

To test for the presence of tannin compounds, add 2 mL of filtrate to a test tube, then add 3 drops of 5% $FeCl_3$ solution and shake gently. If tannin compounds are present in the sample, the color of the solution will turn blackish green or dark blue.

2.5.5 Steroids and Terpenoids

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The test to detect steroid and terpenoid compounds is carried out by adding 3 mL of filtrate to a test tube, then adding 3 drops of anhydrous acid and shaking gently. After that, 2 drops of H_2SO_4 are added and the filtrate mixture is shaken. If there are steroid compounds, the solution will turn green or blue, while for terpenoids, the color of the solution will turn red or reddish purple.

3. RESULTS AND DISCUSSION

In this study, acetone extract from butterfly pea flowers was obtained using the maceration method for 3x24 hours by stirring the sample to accelerate the equilibrium of the concentration of the extracted material in the solvent and replacing the old solvent to prevent solvent saturation, so that the extracted compounds became more optimal [15]. The resulting extract was a dark brownish-green color and weighed 35.21 grams.

3.1 Antibacterial Activity Test

3.1.1 Disk Diffusion Test

In the antibacterial test, the sample used was acetone extract of butterfly pea flower at a concentration of 10,000 µg/mL, with chloramphenicol antibiotic 30 µg as the positive control and DMSO as the negative control. Based on the results of the study, DMSO gave a negative result, no inhibition zone was formed. This proves that DMSO as an extract solvent has no antibacterial activity. Meanwhile, in the disc diffusion test against *P. acne* (ATCC 11828) and *C. freundii* (ATCC 13316) bacteria, the diameter of the inhibition zone was 8.4 mm and 8.3 mm, respectively. The formation of a clear zone around the test extract disc paper on the agar medium was caused by the secondary metabolites contained in the acetone extract of butterfly pea seeds, which were able to inhibit bacterial growth around the test extract. The calculation of the percentage of bacterial inhibition effectiveness of the acetone extract of butterfly pea flowers against *P. acne* bacteria was 34% and against *C. freundii* bacteria was 22.4%. Based on the percentage of bacterial inhibition effectiveness, the acetone extract of butterfly pea flowers had a lower effectiveness against *P. acne* than against *C. freundii*. The results of the calculation of the percentage of bacterial inhibition effectiveness of the acetone extract of telang flowers against *P. acne* bacteria was 34% and *C. freundii* bacteria was 22.4%. Based on the percentage of bacterial inhibition effectiveness < 80%, it is categorized as less effective [4].

Previous studies found that acetone extracts from butterfly pea flowers at four concentrations, namely 5%, 15%, 25%, and 35%, produced inhibition zones measuring 2.73 mm, 5.36 mm, 8.96 mm, and 11.7 mm against *S. aureus* bacteria [2]. Meanwhile, another study found that ethanol extracts of butterfly pea flowers at four concentrations, namely 25%, 50%, 75%, and 100%, produced inhibition zones of 6.3 mm, 6.9 mm, 8.3 mm, and 8.8 mm against ESBL *E. coli* bacteria [30].

3.1.2 MIC (Minimum Inhibitory Concentration) Test using the Microdilution Method

The MIC test was conducted to determine the lowest concentration of the sample capable of inhibiting bacterial growth. This concentration was indicated by visual clarity. The MIC test results are shown in Table 1 below.

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Table 1. MIC Test Results

Sample	MIC (µg/mL)	
	P. acne	***C. freundii***
Chloramphenicol (500 ppm)	18,75	37,5
Butterfly pea flower acetone extract 1% (10.000 ppm)	1250	1250

Based on Table 3.1, the MIC test results show that 1% blue pea flower acetone extract is able to inhibit the growth of *P. acne* and *C. freundii* bacteria at a concentration of 1250 µg/mL. If the MIC value is < 100 µg/mL, the activity is classified as very strong; a value of 625 µg/mL > MIC < 100 µg/mL is classified as moderate; and an MIC value > 625 µg/mL is classified as weak antibacterial activity 10. Therefore, the 1% acetone extract of butterfly pea flower has weak antibacterial activity against *P. acne* and *C. freundii*.

3.1.3 MBC (Minimum Bacteriocidal Concentration) Test using the Microdilution Method

The MBC test was conducted to determine the lowest concentration of extract capable of killing microorganisms. The MBC test results are shown in Table 2 below.

Table 2. MBC Test Results

Sample	MBC (µg/mL)	
	P. acne	***C. freundii***
Chloramphenicol (500 ppm)	125	250
Butterfly pea flower acetone extract 1% (10.000 ppm)	> 5000	> 5000

Based on Table 2, the MBC test results for chloramphenicol were 125 µg/mL against *P. acne* bacteria and 250 µg/mL against *C. freundii* bacteria, meaning that chloramphenicol requires a high concentration, which is 6 times the MIC value. Meanwhile, the MBC value for blue pea flower acetone extract against *P. acne* and *C. freundii* bacteria is > 5000 µg/mL, which means that a concentration greater than 5000 µg/mL is required to kill these two bacteria [5]. Bactericidal and bacteriostatic characteristics are related to how secondary metabolites interact with bacteria.

In another study, water extract of butterfly pea flower (1 mg/mL) obtained an MIC value of 250 µg/mL and an MBC value of 1000 µg/mL against *Staphylococcus aureus* and *Staphylococcus epidermidis* bacteria, an MIC value of 250 µg/mL and an MBC value of 2000 µg/mL against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* bacteria [9]. The results varied depending on the extraction solvent, extract concentration, and test bacteria used. Other factors that caused differences in results were biological, technical, and chemical factors. Biological factors include plant source, growing location, planting method, maintenance, and fertilization, all of which can affect the content of secondary metabolites [32]. Technical testing factors such as intervention during the process, media or culture sterilization, testing duration, and extraction method also play an important role in determining the results [22]. In addition, the chemical factors of the extract itself, namely the type, composition, and quantity of active compounds contained, greatly determine the potential antibacterial activity. The combination of these factors has a direct impact on the quality of the extract, which ultimately affects the effectiveness of the extract as an antibacterial agent [11].

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3.2 Phytochemical Screening Test

Phytochemical screening test is a qualitative test conducted to determine the content of secondary metabolites in acetone extract of butterfly pea flower (*C. ternatea*) using test tubes. In this study, blue pea flower acetone extract was found to contain flavonoids, saponins, tannins, and terpenoids. The results of the phytochemical screening test of blue pea flower acetone extract (*C. ternatea*) are shown in Table 3 below.

Table 3. Phytochemical Screening Test Results

Compound	Reagent	Test Result
Alkaloids	Mayer Dragendorff Wagner	- - -
Flavonoids	Mg + HCl	+
Saponin	Aquadest	+
Tannin	$FeCl_3$ 5%	+
Steroids and Terpenoids	$(CH_3CO)_2O$ + H_2SO_4	+ -

Description :
(+) : Positive
(-) : Negative

The alkaloid compound test showed negative results, indicated by the absence of precipitation in the acetone extract of butterfly pea flowers. The acetone extract of butterfly pea flowers in this study did not contain alkaloid compounds, possibly due to differences in the growing location of the plants. Several studies have shown that acetone extracts of butterfly pea flowers contain alkaloid compounds [31]. The test results showed that the acetone extract of butterfly pea flower was positive for flavonoid compounds, indicated by a change in the color of the solution to orange. In testing flavonoid compounds, the purpose of adding Mg metal and HCl was to reduce the benzopyron nucleus found in the flavonoid structure, thereby forming an orange-colored flavilium salt [27]. The addition of HCl causes an oxidation-reduction reaction, where Mg metal acts as a reducing agent and reacts with flavonoid compounds [17]. The chemical reaction between flavonoid compounds and Mg metal and HCl can be seen in Figure 1 below.

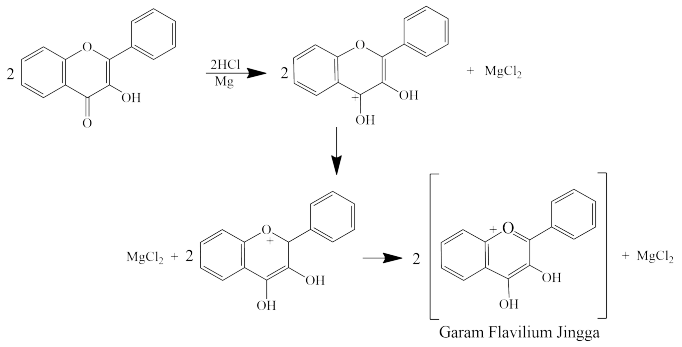

Figure 1. Chemical reaction between flavonoid compounds and Mg metal and HCl [27]

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The saponin compound test yielded positive results and produced stable foam after vigorous shaking. The foam that appeared was caused by the presence of glycosides that are capable of producing foam when dissolved in water, then through a process of hydrolysis, becoming glucose compounds and their aglycones. Positive results from saponin testing were seen in experiments using solvents such as distilled water, acetone, ethanol, and methanol. Saponin compounds have polar and non-polar parts that are surface-active, so when mixed with water and shaken, a hydrolysis process occurs that forms a micelle structure. The micelle structure is formed with the polar groups facing outward and the nonpolar groups facing inward, so that it looks like foam [23]. The hydrolysis reaction of saponin compounds with water can be seen in Figure 2 below.

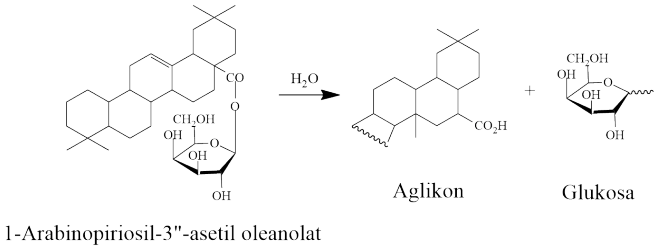

Figure 2. Hydrolysis reaction of saponin compounds with water [14]

The tannin compound test yielded positive results, indicated by a change in the color of the solution to dark green. The phytochemical screening test using 5% $FeCl_3$ aims to detect the presence of phenolic compounds in the sample. If phenolic groups are present, a dark green or dark blue color will appear. If the phytochemical test with $FeCl_3$ shows positive results, it means that there are phenolic compounds in the sample, and one possibility is tannin, which belongs to the polyphenol compound category. The blackish green or ink blue color that appears in the extract after adding $FeCl_3$ is caused by the reaction of tannin compounds with Fe^{3+} ions [8]. The chemical reaction between tannin compounds and $FeCl_3$ can be seen in Figure 3 below.

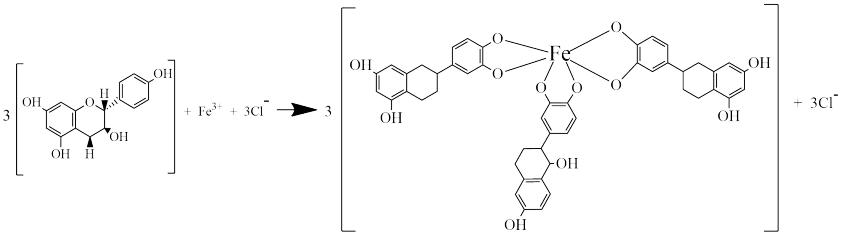

Figure 3. Reaction of tannin compounds with $FeCl_3$ [7]

Steroid compound testing yielded positive results, indicated by a color change to green. The addition of Liebermann-Burchard reagent aims to produce acetyl derivatives from the acetylation reaction on the OH group, which will form a green or blue ring and a red or purple color [14]. The process begins with the release of hydrogen and electron groups, causing a shift in double bonds. The compound undergoes resonance, acting as an electrophile and carbocation. The attack of the carbocation causes electrophilic addition, followed by the release of hydrogen. After that, the hydrogen and electron groups are released, causing the compound to undergo conjugation elongation, which results in the appearance of a red ring [20]. The reaction mechanism of steroid compounds with Lieberman-Burchard reagents can be seen in Figure 4 below.

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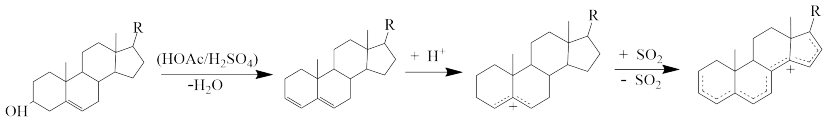

Figure 4. Mechanism of reaction of steroid compounds with Liberman-Burchard reagent [6]

In testing for terpenoid compounds, the results obtained were negative due to a color change to green. This indicates that terpenoid compounds are either absent or their concentration is too low to be detected using this testing method. Positive results are usually indicated by a color change in the solution to purple, orange, or golden yellow [24]. The color change occurs due to the oxidation process in the terpenoid compound, which causes the formation of conjugated double bonds [32].

4. CONCLUSION

The acetone extract of butterfly pea flower showed weak antibacterial activity against *P. acne* and *C. freundii* bacteria with an MIC value of 1250 µg/mL. The acetone extract of butterfly pea flower contains secondary metabolites such as flavonoids, saponins, tannins, and steroids.

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