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Antibacterial Activity of Ethyl Acetate Fraction of a Vicious Combination of Glass and Binahong Against Gram-Negative and Gram-Positive Bacteria Causing Diabetic Ulcers

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The author(s) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ABSTRACT

Purpose: This study aimed to evaluate the antibacterial activity of the ethyl acetate fraction combination of *Strobilanthes crispa* and *Anredera cordifolia* leaves against Gram-positive and Gram-negative bacteria causing diabetic ulcers, namely *Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. It was hypothesized that the 1:1 combination at 75% concentration would inhibit bacterial growth due to its secondary metabolite content.

Research Method: This true experimental study used a one-factor Completely Randomized Design. Antibacterial testing was conducted *in vitro* using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar with a 1:1 combination at 75% concentration, Ciprofloxacin 5 µg as a positive control, and sterile aquadest as a negative control. Data were analyzed using Shapiro-Wilk, One-way ANOVA, and Duncan Multiple Range Test. Phytochemical screening was also performed.

Results and Discussion: The combination extract showed mean inhibition zones of 10.7 mm against *S. aureus*, 8.8 mm against *K. pneumoniae*, and 8.1 mm against *P. aeruginosa*. The inhibition response was strong against *S. aureus* and moderate against the two Gram-negative bacteria. Phytochemical screening identified flavonoids, steroids, and tannins in the combination extract.

Implications: The findings indicate that the extract combination has potential as a natural antibacterial candidate against diabetic ulcer pathogens, although its effectiveness remains below that of Ciprofloxacin. Further *in vivo toxicity* studies and quantitative phytochemical analyses are needed.

Keywords: strobilanthes crispa; anredera cordifolia; diabetic ulcer; antibacterial activity; ethyl acetate fraction; phytochemical screening.

1. Introduction

Diabetes Mellitus (DM) is characterized by blood sugar levels >300 mg/dl hyperglycemia (hyperglycemia) due to abnormal function or insulin secretion (Milita et al., 2018). According to the International Diabetes Federation (IDF), the data of individuals with diabetes globally in 2021 touched 537 million. This number will increase to 783 million people by 2045. According to the IDF, Indonesia occupies the fifth position of the country with the highest number of individuals with diabetes, which is

19.5 million in 2021 which is estimated to continue to increase to 28.6 million people until 2045 (IDF, 2021). Referring to data from the East Kalimantan Provincial Health Office, there was an increase in the number of cases in 2021 with the number of patients reaching 80,672 thousand people (East Kalimantan Health Office, 2021), and in 2022 as many as 88,403 thousand people (East Kalimantan Health Office, 2022).

A common chronic complication of diabetes mellitus (DM) is diabetic ulcers. Diabetic ulcers are complications of diabetes mellitus characterized by open wounds on the surface of the skin, accompanied by necrosis, or death of local tissues (Gaol et al., 2017). Open wounds are particularly susceptible to bacterial exposure. Previous studies on ulcer samples from diabetic patients showed the prevalence of various bacteria, including Gram-positive bacteria, including Staphylococcus aureus (22.5%). Gram-negative bacteria include Pseudomonas aeruginosa (7.5%), and Klebsiella pneumoniae (15%) (Zuliana et al., 2023).

Antibiotics have long been used to treat bacterial infections, but their constant and improper use has raised concerns about bacterial resistance to antibiotics. Another solution to prevent persistent infections and antibiotic resistance is to use natural antibacterial compounds found in medicinal plants. Supriyadi's research (2016) states that the abominable (Strobilanthes crispa (L.) Blume) contains metabolites, such as flavonoids, alkaloids, saponins, and tannins. Binahong leaves (Anredera cordifolia) also have antibacterial compounds. This efficacy is due to the content of metabolites contained in binahong leaves, namely glycosides, polyphenols, flavonoids, alkaloids, tannins, steroids, terpenoids, and saponins (Abidin et al., 2022).

A single extract of Kevil Beling has been shown to have antibacterial activity against the growth of S. aureus bacteria in extracts with concentrations of 10%, 20%, 30% and 40% with inhibition capacity of 2.08 mm, 3.12 mm, 4.02 mm, 5.12 mm mm respectively (Jariyah et al., 2021). Meanwhile, a single extract of binahong has been tested on Gram-negative bacteria, namely E. coli bacteria with concentrations of 0%, 25%, 50%, 75% and 100% which shows that binahong leaves have antibacterial activity against E. coli in vitro (Narulita et al., 2019). However, research on the combination of ethyl acetate fraction extract is still limited, especially on bacteria that cause diabetic ulcers. Therefore, through this study, it can be known the potential to increase the effectiveness of antibacterial with combined extracts.

The selection of a 75% extract concentration in this study aims to maintain a high enough amount of active compounds to still provide optimal biological effects. At this concentration, the extract still contains a small amount of water left over from the previous process which can help diffuse the active compound into the bacterial cell wall structure thereby enhancing the effectiveness of the antibacterial properties of the test extract (Kanarek et al., 2025).

Ethyl acetate solvents are used because they can extract compounds that provide antibacterial activity. This property is what causes ethyl acetate extract to have two solubility properties, namely hydrophilic and lipophilic so that it has optimal polarity. Ethyl acetate will have maximum antimicrobial activity, since for the interaction of an antibacterial compound with bacteria a hydrophilic-lipophilic balance is required (Narulita et al., 2019). To identify secondary metabolite compounds that are successfully attracted by ethyl acetate solvents, it is necessary to test the type of compounds contained in plant extracts, namely phytochemical screening tests.

Based on the description, tests were carried out to determine the activity of the combination of the heinous and binahong combination of the bacteria that cause diabetic ulcers with in vitro antibacterial activity tests and screening of metabolite compound content with phytochemical tests.

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The remainder of this paper is organized as follows. Section 2 provides a literature review and hypothesis development. Section 3 presents the research method and design. Section 4 provides the results and discussion. Section 5 is Concluding Remarks and Recommendations.

2. Literature Review and Hypothesis Development

2.1 Diabetic Ulcers

Diabetic ulcers are a chronic complication in people with Diabetes Mellitus which is characterized by open wounds on the surface of the skin, especially in the leg and lower leg area. Hyperglycemia that lasts for a long time causes immune system disorders, thereby reducing the body's ability to fight infections. Uncontrolled blood glucose levels can impair the function of immune cells such as macrophages, lymphocytes, and monocytes, as well as interfere with the processes of chemotaxis, phagocytosis, antigen presentation, and immune cell activation. In addition, peripheral neuropathy as a complication of diabetes causes decreased sensation or numbness in the extremities, so small wounds are often unnoticed by the patient. Wounds that are not handled properly can develop into ulcers and are at high risk of developing aerobic and anaerobic bacterial infections. Open wounds are very susceptible to exposure to germs and bacteria, coupled with high blood sugar levels will make it easier for bacteria to grow and infect (Zuliana et al., 2023).

2.2 Bacteria That Cause Diabetic Ulcers

Bacteria that are normal flora on human skin such as P. aeruginosa can easily infect open wounds in humans with inadequate body defenses (Umarudin et al., 2023). P. aeruginosa infection has been shown to interfere with healing in diabetic wounds in a manner related to its ability to form biofilms (Goldufsky et al., 2015). Other bacteria such as S. aureus can cause diseases with typical signs such as inflammation, necrosis and the formation of abscesses. The infection can be a light pus-filled lump on the skin that worsens the ulcer condition. The bad thing is that these bacteria have evolved and are resistant to antibiotics, such as Methicillin-Resistant Staphylococcus aureus (MRSA). It is feared that it will become an epidemic in hospitals (Umarudin et al., 2023). K. pneumoniae bacteria are a type of opportunistic pathogenic bacteria of Gram negative that can cause respiratory infections, urinary tract infections, nosocomial infections, and even death of up to 10% in humans. These bacteria are easily found in human body fluids such as blood, urine, and phlegm. K. pneumoniae also has urease and permiase citrate enzymes as well as Extended Spectrum Beta Lactamase (ESBL) enzymes that cause resistance to the antibiotics penicillin, cephalosporin, and aztreonam (Tika et al., 2017).

2.3 Binahong Plant (Anredera cordifolia)

Anredera cordifolia or known as binahong is a herbal plant that originated in East Asia and has long been used in traditional medicine. In Indonesia, this plant was initially better known as an ornamental plant, but its use as a traditional medicine is growing, especially in wound healing. Almost all parts of binahong plants, such as leaves, stems, bulbs, and flowers, have therapeutic potential, but leaves are the most commonly used part because of their relatively high content of active compounds.

Ecologically, binahong can grow on various types of soils, both in lowlands and highlands, with an optimal temperature of 16–27°C, a humidity of 70–100%, and a rainfall of 1,800–2,500 mm per year.

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The leaves of binahong are shaped like hearts or cordata. This plant is light green with a length of 5-10 cm and a width of 3-7 cm. The leaves of binahong look quite thin and weak, at the tips of the leaves look tapered and the base is split, the edges of the leaves are flat but sometimes wavy, and the surface of the leaves is smooth and slippery (Abidin et al., 2022).

The content of secondary metabolites such as glycosides, steroids, terpenoids, alkaloids, saponins, tannins, polyphenols, and flavonoids in binahong leaves acts as antioxidants and anti-inflammatories, which support the tissue regeneration process and accelerate wound healing, including in the case of diabetic ulcers.

2.4 Scarlet Witch (Strobilanthes crispa (L.)Blume)

Strobilanthes crispa (L.) Blume or kevi beling is a herbal plant that is widely found in various regions of Indonesia and is often used as a hedge plant or traditional medicine. This plant can grow at an altitude of 50-1,200 m above sea level. Its height can reach 0.5-1 m. The stems are segmented, round, branched, coarse-haired, and green in color. Leaves with short stems opposite, green, elongated or almost elliptical, serrated, tapered tip, rough surface with a length of 9-18 cm and a width of 3 6 cm (Ramadhani et al., 2021).

This plant is known to have various health benefits because it contains active compounds that are antibacterial, anti-inflammatory, and antioxidant. Oval-leaved leaves contain secondary metabolites such as flavonoids, saponins, tannins, steroids, alkaloids and terpenoids (Abidin et al., 2022). This content has the potential to support the wound healing process by inhibiting the growth of microorganisms and accelerating the formation of new tissues.

3. Research Method

This study is a true experimental study with a one-factor complete random design (RAL) aimed at assessing the antibacterial effectiveness of the ethyl acetate fraction in combination with Anredera cordifolia and Strobilanthes crispa leaf extracts in a ratio of 1:1 concentration of 75% to the growth of Staphylococcus aureus, Klebsiella pneumoniae, and Pseudomonas aeruginosa in vitro by disc diffusion method Kirby–Bauer on Mueller Hinton Agar (MHA) media, using a 5 µg/mL positive control of siproflocacin and a sterile aquadest negative control . The number of repetitions is determined based on Federer's formula so that nine repetitions are obtained in each treatment (a total of 27 experimental units). Leaf samples were obtained through simple random sampling with the criteria of inclusion of fresh leaves, free of pests and diseases, then simplicia were made, macerated using ethyl acetate, evaporated with a rotary evaporator, and tested solvent-free before making a combination solution. The bacterial suspension was adjusted to the McFarland standard of 0.5 and then inoculated at MHA, the disc paper was dripped with 15 µL of extract and incubated at 37°C for 18–24 hours, then the diameter of the inhibition zone was measured in mm. In addition to antibacterial tests, qualitative phytochemical screening is performed to identify secondary metabolites (alkaloids, flavonoids, tannins, saponins, steroids/terpenoids). The data were analyzed through the Shapiro–Wilk normality test, followed by One-way ANOVA and DMRT follow-up test if $p < 0.05$, and the percentage of inhibition and antibacterial effectiveness was calculated based on the comparison of the diameter of the extract inhibition zone with the positive control.

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4. Results and Discussion

4.1 Analysis Results

In this study, an antibacterial effectiveness test was carried out on the antibacterial effectiveness of a combination of binahong and kevi beling leaves with a concentration of 75% in a 1:1 ratio against the bacteria Staphyloccus aureus ATCC 25923, Klebsiella pneumoniae ATCC 27736, and Pseudomonas aeruginosa ATCC 27853 using the Kirby Bauer disc diffusion method. Testing was conducted with single extracts and combination extracts with a concentration of 75% and a 1:1 ratio of combination extracts.

4.1.1 Average Diameter of Inhibitory Zones of Single Extracts and Combinations of Pesticides and Pesticides on the Growth of S. aureus, K. pneumoniae and P. aeruginosa Bacteria

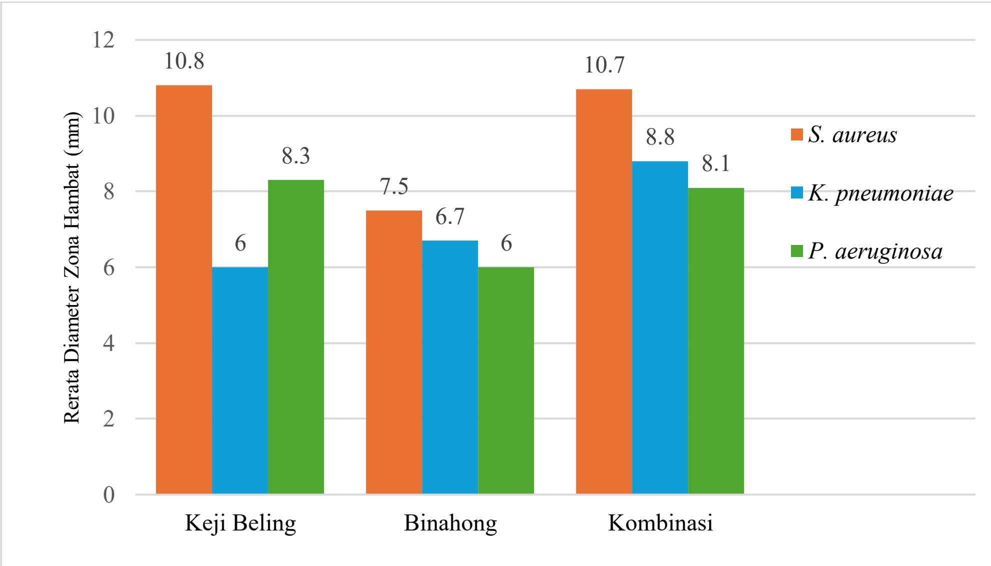

Figure 1. Chart of Average Diameter of Inhibition Zone

The test results showed that there was inhibition of bacterial growth in a single extract of Kevi beling, the largest inhibition in S. aureus bacteria was 10.8 mm and the smallest in K. pneumoniae bacteria was 6 mm. In a single extract of binahong there was inhibition against bacteria, the largest in S. aureus, which was 7.5 mm and the smallest in P. aeruginosa, which was 6 mm. The combination extract obtained the largest average resistance in S. aureus bacteria which was 10.6 and the smallest average in P. aeruginosa which was 8.1 mm. Based on the average diameter of the inhibition zone of the combination of the heinous ethyl acetate fraction and binahong ratio of 1:1 with a concentration of 75%, it shows that combination extracts in S. aureus, K. pneumoniae, and P. aeruginosa bacteria have the ability to inhibit bacterial growth.

4.1.2 Resistance Response of Combination of Ethyl Acetate Fraction Extract Combination to S. aureus, K. pneumoniae and P. aeruginosa Bacteria

Classification was carried out on the inhibition response in each bacteria. The results of the category of bacterial inhibition response are as follows:

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Table 1. Impediment Response

Bacteria	Average Diameter of Inhibition Zone (mm)	Category Impediments
S. aureus	10.7	Strong
K. pneumoniae	8.8	Medium
P. aeruginosa	8.1	Medium

Based on Table 1, it can be seen that the bacterial inhibition response to the combination of the heinous and binahong extracts with a 75% ethyl acetate fraction has a moderate inhibition response (5-10 mm) against K. pneumoniae and P. aeruginosa bacteria, to strong (>10 mm) against S. aureus bacteria (Santoso et al., 2020).

4.1.3 Antibacterial Inhibition Zone Activity of Ethyl Acetate Fraction A Combination of Harmful and Harmful Substances Against S. aureus, K. pneumoniae and P. aeruginosa Bacteria

The determination of bacterial inhibition activity is carried out based on the inhibition data obtained and compared between the diameter of the inhibition zone and the diameter of the disk used in the form of a percentage (%).

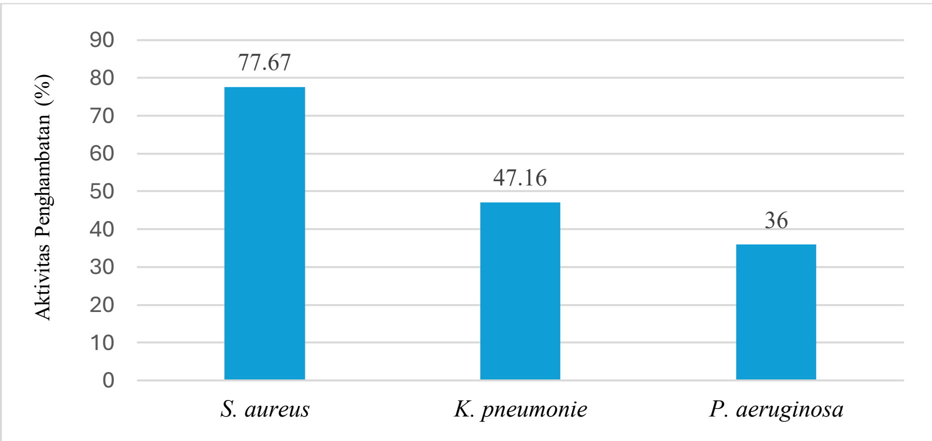

Figure 2. Inhibition Activity Graph

Based on Figure 2, the combination of the odious extract and binahong of the ethyl acetate fraction was a ratio of 1:1 with a concentration of 75% against bacteria S. aureus, K. pneumoniae and P. aeruginosa has the largest percentage of inhibitory activity in bacteria S. aureus with a percentage of 77.67% and the smallest inhibition activity in bacteria P. aeruginosa with a percentage of 36%.

4.1.4 Antibacterial Effectiveness of Ethyl Acetate Fraction of a Combination of S. aureus and Binahong Against the Growth of S. aureus, K. pneumoniae and P. aeruginosa Bacteria

Based on the test results, it was known that the diameter of the largest inhibition zone in the positive control of the antibiotic Ciprofloxacin 5µg was 39.5 mm in P. aeruginosa bacteria, 35 mm in K. pneumoniae bacteria and 34 mm in S. aureus bacteria which were included in the very strong inhibition category. To determine the antibacterial effectiveness of the combination of Kevi Beling and binahong extracts, a comparison was made between the diameter of the largest inhibition zone of the combination of Kevi Beling and binahong extracts in a ratio of 1:1 (mm) and the diameter of the positive control inhibitory zone of Ciprofloxacin 5µg and then multiplied by 100%.

Table 2. Antibacterial Effectiveness Against Antibiotics Ciprofloxacin 5µg

Test Bacteria	Antibacterial Effectiveness of Extract (%)
Staphylococcus aureus	36.76%
Klebsiella pneumoniae	27.14%
Pseudomonas aeruginosa	22.78%

The results of this study show that the combination of the odious and binahong extract of the ethyl acetate fraction with a concentration of 75% in a ratio of 1:1 has the potential as a natural antibacterial against S. aureus, P. aeruginosa and K. pneumoniae bacteria. However, this extract is no more effective when compared to the antibiotic Ciprofloxacin 5µg.

4.1.5 Effect of Ethyl Acetate Fraction of a Combination of Harmful and Binahong on Inhibition of Growth of S. aureus, K. pneumoniae and P. aeruginosa Bacteria

Based on the results of the one-way ANOVA statistical test, single extract and combination (1:1) concentration of 75% had a value of ($p>0.05$) which meant that the single extract and the combination were significant against the growth inhibition of S. aureus, P. aeruginosa and K. pneumoniae bacteria and H0 were rejected. In the follow-up statistical test of Duncan's Multiple Range Test (DMRT), a combination of 75% concentration of heinous leaves and binahong with a ratio of 1:1 yielded the largest diameter of inhibition zone in K. pneumoniae, while the 75% concentration of the heinous single extract produced the largest diameter of the average inhibition in S. aureus and P. aeruginosa.

4.1.6 Phytochemical Screening Test Results Identification of Antibacterial Secondary Metabolite Compounds Combination Extract and Ethyl Acetate Fraction

The extracts used in this test were a single extract of the vile beling, a single extract of binahong and a combination of the extract of the vile and binahong in a ratio of 1:1. Based on the results of the test or phytochemical screening, the following results were obtained:

Table 3. Phytochemical Screening Test Results

Yes	Compound Groups	Observation Results		
		Scarlet Witch	Stuttgart	Combinations
1	Alkaloids	-	-	-
2	Flavonoids	+	+	+
3	Tannins	-	+	+
4	Saponins	-	-	-
5	Steroids	+	+	+
6	Terpenoids	-	-	-

(+) : Identified; (-) : Unidentified

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Based on Table 3, the phytochemical screening results of single extracts and combinations of ethyl acetate fractions showed, of the 6 groups of compounds tested, there were 3 compounds that produced positive results in single binahong extracts and combination extracts, namely flavonoid compounds, steroids and tannins. Meanwhile, in the heinous single extract, the ethyl acetate fraction contains 2 compounds, namely flavonoids and steroids.

4.2 Discussion

Based on these results, it shows that various variations of resistance are formed, namely having a moderate (5-10 mm) to strong (>10 mm) inhibitory response from both single extracts and combination extracts. The inhibition zone formed indicates that a single extract and a combination extract of ethyl acetate with a concentration of 75% have an inhibitory effect on S. aureus, K. pneumoniae, and P. aeruginosa bacteria. Meanwhile, the variation of resistance varies due to the compound content in each extract and the ability of bacteria to fight antibacterial compounds with different mechanisms of action.

The peptidoglycan layer on the cell wall of Gram-positive bacteria is thick and rigid, while the peptidoglycan layer on the cell wall of Gram-negative bacteria is thinner and flexible, consists of one or more peptidoglycan layers, contains high lipids and has an additional barrier in the form of outer membranes and other defense systems so that the active compound is more easily attacked by Gram-positive bacteria than Gram-negative bacteria (Sholihah et al., 2024). In the phytochemical screening test of the extract of kevi beling and binahong ethyl acetate fraction against 5 compounds that have been tested, only 3 compounds showed positive results, namely flavonoid compounds, steroids and tannins. This is due to the compatibility of the polarity between these compounds which are semipolar to slightly polar with the characteristics of ethyl acetate as a semipolar solvent, thus allowing extraction to take place optimally. In contrast, alkaloid compounds and saponins are not detected because they both have a higher polarity.

The mechanism of action of flavonoid compounds on bacteria is by damaging cell membranes, interfering with nucleic acid synthesis and binding to cell wall proteins. This compound works more effectively on Gram-positive bacteria, such as S. aureus, which have a thick peptidoglycan layer and are not protected by the outer membrane. In contrast, in Gram-negative bacteria such as P. aeruginosa and K. pneumoniae, flavonoids have limited access due to the presence of an outer membrane layer that protects the cell wall (M. Guli et al., 2024). The content of steroids and tannins in the extract can damage the cell membrane of bacteria, which also work more effectively on Gram-positive bacteria (Rais Khasanah et al., 2021).

The existence of inhibiting activities that have been formed and also the results of the calculation of effectiveness in the combination of kevi beling and binahong extracts with a ratio of 1:1 at a concentration of 75% against the growth of S. aureus, P. aeruginosa and K. pneumoniae varied, show that the bioactive compounds in the extracts of kevi beling and binahong have high antibacterial activity. The ethyl acetate fraction has potential and works in a synergistic in inhibiting bacterial growth however, this extract is no more effective when compared to the antibiotic Ciprofloxacin 5µg.

Based on the results of the One way ANOVA and Duncan's Multiple Range Test (DMRT) statistical tests, it was found that the single extract and a combination (1:1) concentration of 75% had a value of ($p>0.05$) which can be interpreted as a single extract and a significant combination to inhibit the growth of the test bacteria. A combination of 75% concentration of 75% and a 1:1 ratio of a combination of 75% of the bacteria produced the greatest influence on K. pneumoniae bacteria while

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a single extract of 75% concentration of S. aureus and P. aeruginosa bacteria produced the greatest influence on S. aureus and P. aeruginosa bacteria.

5. Concluding Remarks and Recommendation

Based on the research that has been conducted, the combination of kevi beling extract and binahong ethyl acetate fraction ratio of 1:1 concentration of 75% has an average inhibition zone that varies with moderate to strong inhibition categories. The inhibition zone formed indicates that the combination extract of ethyl acetate with a concentration of 75% has an inhibitory effect on S. aureus, K. pneumoniae, and P. aeruginosa bacteria due to the content of secondary metabolites, namely flavonoid compounds, steroids, and tannins. These compounds have an effect on damaging the cell walls of bacteria, especially in Gram-positive bacteria. The existence of inhibiting activity that has been formed and also the results of the effectiveness calculation of the combination of keling and binahong extracts with varying results show that the bioactive compounds in the ethyl acetate fraction extract have the ability and work synergistically in inhibiting the growth of bacteria, however, this extract is not more effective when compared to the antibiotic Ciprofloxacin 5µg.

This study is limited to in vitro testing and quantitative phytochemical analysis has not been carried out to determine the level of active compounds in the fraction used. Further research is needed to evaluate the effectiveness in vivo as well as conduct toxicity tests to support therapeutic development and also quantitative phytochemical tests to determine the level or quantity of active compound content contained in the test extract.

Statement of Use of Generative AI

During the preparation of this work, the author used generative artificial intelligence tools to support the scientific writing process. Grammarly was used to check grammar, refine writing style, and improve clarity in scientific writing. All interpretations, analyses, and conclusions presented in this study are the sole responsibility of the author.

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