

Antibacterial Activity Test of a Combination of Ethanol Extract of Bidara Leaf (*Ziziphus mauritiana*) and Betel Leaf (*Piper betle L*) Against *Staphylococcus aureus* in Vitro

Azka Nadya Robbani^{1*}, I Putu Dedy Arjita², Deny Sutrisna Wiatma³, Ety Retno Setyowati⁴

^{1,2,3,4} Universitas Islam Al-Azhar, Indonesia

* Corresponding Author:

Email: azka.nadyar@gmail.com

Abstract.

Infections caused by *Staphylococcus aureus* remain a global health problem, particularly due to increasing antibiotic resistance that limits the effectiveness of conventional therapy. This condition encourages the development of alternative antibacterial agents based on natural materials. Bidara leaves (*Ziziphus mauritiana*) and betel leaves (*Piper betle L.*) are known to have antibacterial activity, however the potential of their combination against *Staphylococcus aureus* has not been widely studied. This study aims to evaluate the antibacterial activity of a combination of ethanol extracts of bidara leaves and betel leaves against the growth of *Staphylococcus aureus* with three variations of concentration ratios, namely 100%:0%, 50%:50%, and 0%:100%. There are also 2 control groups, namely a positive control using the antibiotic Clindamycin and a negative control using 10% DMSO. The study used a true experimental design with a post-test only control group design and was conducted using an inhibition zone test with the disc diffusion method. In addition, phytochemical screening tests were carried out to identify active compounds in each extract. The results of phytochemical screening of bidara leaf extract, betel leaf extract, and their combination showed positive results containing secondary metabolites in the form of alkaloids, flavonoids, tannins, terpenoids, and phenols. The results showed that the average diameter of the inhibition zones formed were group 1 (100%:0%) 25.6 mm, group 2 (50%:50%) 19.4 mm, and group 3 (0%:100%) 26.2 mm. The Mann-Whitney test showed significant differences between groups ($P=0.000$), however further tests showed that there were no significant differences between the treatment groups. In conclusion, the combination of bidara leaf (*Ziziphus mauritiana*) and betel leaf (*Piper betle L.*) extracts has antibacterial activity against *Staphylococcus aureus*, but does not show a synergistic effect and is not more effective than single extracts.

Keywords: Antibacterial; Bidara Leaf Extract; Betel Leaf Extract and *Staphylococcus aureus*.

I. INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that is often found on the skin and nose of about 30% of the human population. Generally, these bacteria are harmless, but they can sometimes cause infections (Hanina *et al.*, 2022). Infections caused by *Staphylococcus aureus* are still a major problem in the global health sector, as this bacterium has been identified as the leading cause of death due to bacterial infections in 135 countries, as well as being the highest cause of death in the age group over 15 years globally (Ikuta *et al.*, 2022). Some of the diseases caused by *Staphylococcus aureus* include *Staphylococcal scalded skin syndrome*, osteomyelitis, abscesses, bacteremia, endocarditis, toxic shock syndrome, and skin infections such as furunkel (Pratiwi *et al.*, 2022). The high prevalence of *Staphylococcus aureus* infections is a serious concern because this bacterium not only causes various infectious diseases, but also has a high potential for resistance to antibiotics. In response to this, the WHO in 2017 included *Staphylococcus aureus*, specifically *Methicillin-Resistant Staphylococcus aureus* (MRSA), in the list of pathogenic bacteria that require the development of new antibiotics, because they are resistant to common antibiotics (Zahara *et al.*, 2024). This situation is in line with the increasing threat of antimicrobial resistance globally, which the WHO estimates could lead to up to 10 million deaths by 2050 if not addressed immediately (Putri *et al.*, 2023). In fact, globally, resistant bacterial infections have contributed to about 4.95 million deaths in 2019 (Gach *et al.*, 2024).

In Indonesia alone, the prevalence of antibiotic resistance cases continues to increase, with around 1.27 million people dying each year from infections that are not responsive to medications, which are exacerbated by the improper use of antibiotics, especially during the pandemic (Zahara *et al.*, 2024). Based on this, other alternatives are needed as a substitute for antibiotics to prevent resistance and side effects of therapy. One alternative therapy to replace antibiotics is to use natural ingredients, in the form of the use of herbal plants that are easy to find in the surrounding environment. One of the natural antibacterial that can be

used as an alternative treatment is the Bidara Plant (*Ziziphus mauritiana*) and the Betel Plant (*Piper betle L.*). Bidara, scientifically known as *Ziziphus mauritiana*, originates from the West Asian region and has long been used in Traditional Chinese Medicine to treat various diseases, such as urinary tract disorders, digestion, fever, liver disorders, weakness, anemia, obesity, diabetes, bronchitis, skin infections, loss of appetite, pharyngitis, diarrhea, cancer, and insomnia (Qudsiyyah, 2021). In Indonesia, bidara leaves are known as herbal plants that have various pharmacological properties, especially in the field of antimicrobials. The leaves contain various secondary metabolite compounds such as flavonoids, tannins, saponins, and phenols that act as antibacterial agents.

Each of these active compounds has a specific mechanism of action in inhibiting growth and killing bacteria. Flavonoids interfere with the function of bacterial cell walls through the formation of complexes with extracellular proteins and inhibit bacterial motility. Tannins are known to inhibit bacterial enzymes as well as damage cell membranes through their toxic properties. Saponins work by lowering the surface tension and damaging the permeability of the cell membrane until the cell dies. Phenols play a role in inactivating enzymes and denatured bacterial cell proteins, which ultimately leads to cell lysis (Shufyani & Dominika, 2022; Aisyah *et al.*, 2020; Sadiyah *et al.*, 2022). The betel leaf, or the plant scientifically known as *Piper betle L.*, is a medicinal plant native to tropical Asia and East Africa, and is widespread in Indonesia, Thailand, Malaysia, India, Sri Lanka, and Madagascar. This plant is empirically known to have various properties in traditional medicine, such as stopping bleeding, relieving itching, treating canker sores, as well as curing infections caused by bacteria and fungi. Green betel leaves are known to have antibacterial activity against various Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*. This antibacterial activity is supported by the content of various active compounds such as alkaloids, flavonoids, saponins, tannins, and terpenoids, each of which has a specific mechanism of action in inhibiting the growth and killing of bacteria.

Alkaloids work by disrupting the peptidoglycan component in bacterial cells so that the cell wall does not form intact and causes cell death. Flavonoids interfere with the function of bacterial cell walls through the formation of complexes with extracellular proteins and inhibit bacterial motility. Saponins work by lowering the surface tension and damaging the permeability of the cell membrane until the cell dies. Tannins are known to inhibit bacterial enzymes as well as damage cell membranes through their toxic properties. In addition, terpenoid compounds can inhibit the growth of cell walls by damaging porines (transmembrane proteins) (Sadiyah *et al.*, 2022). Various studies have supported the antibacterial effectiveness of green betel leaf extract against *Staphylococcus aureus*. Research conducted by Mariadi (2024) showed the antibacterial effectiveness of betel leaf ethanol extract with low concentrations, namely 5%, 10%, 15%, 20%, and 25%, which resulted in an inhibition zone of 3.75 mm (weak), 4.01 mm (weak), 5.32 mm (medium), 5.73 mm (medium), and 6.29 mm (moderate) with a comparator, chloramphenicol. Recent research by Afriliana *et al.*, (2025) also reinforces previous findings, where the concentration of green betel leaf ethanol extract of 40%, 50%, and 60% resulted in inhibition zones of 18.87 mm (strong), 19.75 mm (strong), and 19.87 mm (strong) with tetracycline antibiotic comparators.

The selection of a combination of ethanol extracts of bidara leaf (*Ziziphus mauritiana*) and betel leaf (*Piper Betle L.*) in this study was carried out to explore the synergistic potential of the two extracts, because the combination of active compounds from two different plants has the opportunity to produce stronger antibacterial activity than single use while reducing the risk of bacterial resistance by presenting more than one inhibition mechanism. However, until now, there has been no study that specifically examined the combination of ethanol extracts of bidara leaves (*Ziziphus mauritiana*) and betel leaves (*Piper betle L.*) in inhibiting *Staphylococcus aureus* *in vitro*. This is a research gap that needs to be filled to find out whether the combination of these two extracts can provide a more significant antibacterial effect than their use separately. Therefore, this study was conducted to evaluate the antibacterial activity of a combination of ethanol extracts of bidara leaves (*Ziziphus mauritiana*) and betel leaves (*Piper betle L.*) scientifically and systematically against *Staphylococcus aureus* as an effort to develop herbal antimicrobial agents that are more effective, sustainable, and have the potential to be an alternative treatment in the midst of increasing antibiotic resistance.

II. METHODS

This study is a type of True Experiment research with a post test only control group design using the disc diffusion method to see the effect of the combination of bidara leaf extract (*Ziziphus mauritiana*) and betel leaf (*Piper betle* L) on the diameter of the inhibition zone of *Staphylococcus Aureus* bacteria. The design used in this study was a Complete Random Design (RAL) with 3 treatment groups consisting of a combination of bidara leaf extract (*Ziziphus mauritiana*) and betel leaf (*Piper betle* L) with a concentration ratio of 100%:0%, 50%:50%, 0%:100%. The samples used in this study were bidara leaves (*Ziziphus mauritiana*) and betel leaves (*Piper betle* L) on the island of Lombok, NTB province. *Staphylococcus aureus* bacteria were obtained at the NTB Provincial Health Testing and Calibration Laboratory. The tools used in this study were aluminum foil, sieve, beaker glass, blender, measuring cup, laminari flow, spirit lamp, analytical balance, ose, ruler, petridish, pipette, sterile cotton swab, dark glass bottle, caliper, stirring rod, centrifuge instrument, rack and test tube, petri dish, tissue, densitometer, mask and handsoon.

The materials used in this study were bidara leaves (*Ziziphus mauritiana*), betel leaves (*Piper betle* L), 96% ethanol, McFarland unit turbidity standard, MHA media, *Staphylococcus aureus*, DMSO 10% empty discs and Clindamycin discs 2 µg/disk. The first step in data analysis is to conduct a normality test to find out whether the data is distributed normally or not. Next, a variance homogeneity test was carried out using the Levene test. The data is said to be homogeneous if the significance value ($p > 0.05$). If the data is distributed normally and homogeneously, the analysis is continued using the One-Way ANOVA (Analysis of Variance) test. If the results of the One-Way ANOVA test show a significant difference ($p < 0.05$), then it is followed by a Post Hoc test to find out the difference between treatment groups. Conversely, if the data is not normally distributed, then the analysis is performed using the non-parametric Kruskal Wallis test, and if it shows a significant difference, it is followed by the Mann Whitney test as a follow-up test. The entire data analysis process was carried out using SPSS software version 22 (Nuryadi et al., 2017).

III. RESULT AND DISCUSSION

Ethanol activity tests have been carried out on ethanol extract of bidara leaves (*Ziziphus mauritiana*) with a concentration of 100%, ethanol extract of betel leaves (*Piper betle* L.) with a concentration of 100%, and a combination of ethanol extract of betara leaves (*Ziziphus mauritiana*) and green betel leaves (*Piper betle* L.) with a concentration ratio of 50%; 50%, and the negative control group in the form of DMSO 10% and the positive control group in the form of *Clindamycin*. This research was carried out in November 2025 and was carried out in several locations according to the stages of the research. The manufacture of leaf extracts is carried out at the Integrated Laboratory of the Faculty of Medicine, Al-Azhar Islamic University, Mataram. The extract phytochemical test was carried out at the Laboratory of the Faculty of Mathematics and Natural Sciences, University of Mataram (FMIPA), while the antibacterial activity test was carried out at the West Nusa Tenggara Province Health Testing and Calibration Laboratory Center.

The research stage begins with the preparation of ingredients in the form of bidara leaves (*Ziziphus mauritiana*) and green betel leaves (*Piper betle* L.) which include the process of sorting, washing, drying, and refining until simplicia is obtained. Furthermore, an extraction process was carried out using ethanol solvent to obtain 100% bidara leaf ethanol extract and 100% green betel leaf ethanol extract. The resulting extract is then combined with a concentration ratio of 50%:50%. Phytochemical tests were conducted on bidara leaf ethanol extract, green betel leaf ethanol extract, and a combination of the two extracts to identify secondary metabolite content. The compounds tested included alkaloids, flavonoids, tannins, saponins, terpenoids, steroids and phenols. This phytochemical test is qualitative and aims to provide a preliminary picture of bioactive compounds suspected to play a role in antibacterial activity. The antibacterial activity test was carried out against *Staphylococcus aureus* bacteria using the disc diffusion method by measuring the diameter of the inhibition zone formed around the disc. The data from the measurement of the inhibition zone was then statistically analyzed using SPSS 22 software.

Phytochemical Tests

Phytochemical tests were carried out qualitatively to identify the active compounds contained in bidara leaf extract, betel leaf and combination with ethanol solvents. The results of phytochemical tests on betel leaves are in table 1.

Table 1. Results of Phytochemical Screening Test of Bidara Leaf Extract (*Ziziphus mauritiana*), Betel Leaf (*Piper betle L*) and Combination of Bidara Leaf Extract (*Ziziphus mauritiana*) and Betel Leaf Extract (*Piper betle L*)

Secondary Metabolic	Reagents	Test Results			Positive Results On Literature
		Bidara Leaf	Betel Leaves	Combinations	
Alkaloids	Dragendorff	+	+	+	Reddish-brown deposits
		(Reddish brown deposit)	(Chocolate deposits)	(Chocolate deposits)	
	Mayer	+	+	+	White deposits
		(White deposit)	(White deposit)	(White deposit)	
	Wagner	+	+	+	Reddish-brown deposits
		(Reddish brown deposit)	(Reddish brown deposit)	(Reddish brown deposit)	
Saponins	Aquadest	-	-	-	Foam formed
		(No foam)	(No foam)	(No foam)	
Flavonoids	Concentrated Mg+HCl powder	+	+	+	Red, yellow or orange
		(Brick-orange)	(Orange)	(Brick-orange)	
Tannins	$FeCl_3$	+	+	+	Blackish/green beyond
		(Dark green)	(Dark green)	(Dark green)	
Terpenoids	Chloroform, + Anhydrous acetate + concentrate H_2SO_4	+	+	+	Brownish rings
		(Brownish ring)	(Brownish ring)	(Brownish ring)	
Steroids	Chloroform, + Anhydrous acetate + concentrate H_2SO_4	-	-	-	Bluish-green cicin
		(No bluish-green rings formed)	(No bluish-green rings formed)	(No bluish-green rings formed)	
Phenol	NaCl+ $FeCl_3$	+	+	+	Blackish-blue/dark green
		(Dark green)	(Dark green)	(Dark green)	

Description: + = Positive, - = Negative

Based on the results of phytochemical tests, bidara leaf extracts, betel leaves, and a combination of the two showed positive results containing secondary metabolites in the form of alkaloids, flavonoids, tannins, terpenoids, and phenols, while saponin and steroid compounds were not detected. These results suggest that all three extracts contain a variety of secondary metabolites that are known to have potential biological activity, so they are thought to contribute to the antibacterial activity of each extract.

Antibacterial Activity Test

A combination of ethanol extract of bidara leaves and betel leaves has been tested with a concentration variation of 100%:0%, 50%:50% and 0%:100%, as well as a negative control group in the form of DMSO 10% and a positive control in the form of *Clindamycin*.

Table 1. Antibacterial Test Results of Bidara Leaf Extract Combination (*Ziziphus mauritiana*) and Betel Leaf (*Piper Bethle L*) against *Staphylococcus aureus*

Treatment Groups	Obstacle Zone Area (mm)					Average (mm)	CLSI Interpretation (2024)	Meaning of p-value
	1	2	3	4	5			
Group 1 (100%:0%)	27	27	31	22	21	25,6	Sensitive	0,516
Group 2 (50%:50%)	23	19	26	18	11	19,4	Intermediate	0,854
Group 3 (0%:100%)	25	30	32	22	22	26,2	Sensitive	0,257
(+) Controls	40	40	40	40	40	40	Sensitive	-
Control (-)	0	0	0	0	0	0	Resistant	-

Description:

Group 1 = Combination of bidara leaf extract (*Ziziphus mauritiana*) and betel leaf (*Piper betle L*) with a concentration ratio of 100%:0%

Group 2 = Combination of bidara leaf extract (*Ziziphus mauritiana*) and betel leaf (*Piper betle L*) with a concentration ratio of 50%:50%

Group 3 = Combination of bidara leaf extract (*Ziziphus mauritiana*) and betel leaf (*Piper betle L*) with a concentration ratio of 0%:100%

Control (+) = *Clindamycin antibiotic*

Control (-) = DMSO 10%

Group 1 combination of betel leaf extract: betel leaf extract (100%:0%) and group 3 combination of bidara leaf extract: bidara leaf extract (0%:100%) had an average inhibition zone diameter of 25.6 mm and 26.2 mm, respectively, so it was included in the sensitive category (≥ 21 mm). Meanwhile, group 2 of bidara leaf extract combinations: bidara leaf extract (50%:50%) showed an average inhibitory zone diameter of 19.4 mm which was included in the intermediate category (15–20 mm). Negative control in the form of DMSO 10% did not show the formation of an inhibition zone, so it was declared to have no antibacterial activity. In contrast, the positive control of clindamycin showed the largest inhibitory zone diameter with a value of 40 mm which confirmed the effectiveness of the test method as well as the sensitivity of *Staphylococcus aureus* to the antibiotic. Statistical test analysis was carried out using SPSS software. All data obtained were entered into SPSS and normality tests were carried out using *Shapiro-wilk* because the number of samples was less than 50. The significant value in the normality test showed that all treatment groups had a *p-value* of > 0.05 so that it can be concluded that the inhibitory zone diameter data in the three treatment groups was normally distributed. Next, a variance homogeneity test was carried out using *the Levene test*.

The test results showed a significance value of 0.002 ($p < 0.05$), which indicates that the variance between treatment groups was not homogeneous. Because the assumption of variance homogeneity is not met ($p < 0.05$), the *One Way ANOVA* parametric test cannot be used. In addition, there are groups with zero variance, i.e. positive controls and negative controls, so *the Welch ANOVA test* cannot be performed. Therefore, the analysis of the difference in the diameter of the barrier zone between the treatment and control groups was carried out using the non-parametric *Kruskal Wallis test*. The results of the non-parametric *Kruskal Wallis* test showed a significance value of $p = 0.000$. Because the *p-value* < 0.05 , it can be concluded that there is a significant difference between at least two groups of combined treatment of bidara leaf extract and betel leaf to the diameter of the *Staphylococcus aureus* inhibition zone. To determine which groups are different from each other, *post hoc* analysis can be carried out using *the Mann Whitney test* to find out which groups are significantly different from each other.

Table 2. Test Results *Mann Whitney* between Each Group

Groups	Results	Analysis
Bidara with betel	0,750	No difference
Bidara with Combinations	0,075	No difference
Combination with betel nut	0,116	No difference

Based on the results of *the Post Hoc Mann Whitney follow-up test* conducted to determine the difference in the diameter of the barrier zone between the treatment groups, it was found that there was no statistically significant difference between the bidara leaf extract group, betel leaf extract, and a combination of the two. The comparison between bidara leaf extract and betel leaf extract showed a significance value of $p = 0.750$ ($p > 0.05$), which means that there was no statistically significant difference between the two groups. The very small difference in the average diameter of the inhibition zone shows that the antibacterial ability of bidara leaf extract and betel leaf extract is relatively equivalent. These results indicate that both types of extracts have comparable bacterial inhibition potential, in the absence of any obvious advantages of either treatment. The comparison between bidara leaf extract and the combination of extracts showed a significance value of $p = 0.075$ ($p > 0.05$), which indicates that there was no statistically significant difference in the diameter of the barrier zone produced by the two treatments. Although single bidara leaf extract numerically resulted in a larger average inhibitory zone than combination extracts, the difference was not strong enough to be stated as a noticeable increase in antibacterial effectiveness.

This indicates that the addition of bidara leaf extract to the combination extract has not been able to provide a significant additional effect in inhibiting the growth of bacteria. Furthermore, the comparison between betel leaf extract and the combination of extracts also showed similar results, with a significance value of $p = 0.116$ ($p > 0.05$), meaning that there was no statistically significant difference between the two

treatments. Although a single betel leaf extract numerically resulted in an average greater inhibition zone than a combination extract, the difference was not strong enough to be declared a noticeable increase in antibacterial effectiveness. This indicates that the addition of betel leaf extract to the combination extract has not been able to provide a significant additional effect in inhibiting the growth of bacteria. Overall, the results of the comparison between treatment groups showed that the use of a combination of bidara leaf and betel leaf extracts did not show significant synergistic effects, and the antibacterial effectiveness was relatively equivalent to that of each single extract.

Table 4. Mann Whitney Test Results between Treatment Groups and Positive Controls

Groups	Results	Analysis
Bidara with Positive Control	0,005	There is a difference
Betel with Positive Control	0,005	There is a difference
Combination with Positive Control	0,005	There is a difference

Furthermore, the results of *the Mann Whitney* test between each treatment group with positive controls showed a *value of p* = 0.005 in all comparisons. The *p* < value of 0.05 showed that there was a significant difference between the bidara, betel and combination groups with positive controls. The positive control used is *Clindamycin* which is a standard antibiotic that has a stronger and consistent inhibition of bacterial growth, resulting in a significantly different antibacterial effect than plant extracts. This difference suggests that although bidara, betel extract, and their combinations have antibacterial activity, their antibacterial potential is still lower than that of positive controls. Positive controls are used as a comparison of effectiveness, so this significant difference confirms that the test method used has been valid.

Table 3. Test Results *Mann Whitney* between Treatment Groups with Negative Controls

Groups	Results	Analysis
Bidara with Negative Control	0,005	There is a difference
Betel nut with Negative Control	0,005	There is a difference
Combination with Negative Control	0,005	There is a difference

The results of *the Mann Whitney* test between the treatment and negative control groups showed a *value of p* = 0.005 in the entire comparison (*p* < 0.05), indicating a significant difference between the biden group, betel nut, and combination with the negative control. These findings suggest that all treatment groups have antibacterial activity, as they are able to produce significantly different inhibitory zones compared to negative controls. Negative controls do not contain active antibacterial compounds so they are not able to inhibit bacterial growth or produce only minimal inhibition zones. Thus, this significant difference confirms that the barrier zone formed in the treatment group is a direct result of the activity of the antibacterial compounds contained in the extract, not caused by solvents or other technical factors.

Discussion

Phytochemical Tests

The first phytochemical test is the alkaloid test. The purpose of this test is to determine whether or not alkaloid compounds contain using Dragendorff, Mayer and Wagner reagents. The results of the alkaloid test that have been carried out, bidara leaf extract, betel leaf, and a combination of positive (+) results formed reddish-brown deposits in the dragendorff and wagner tests and white deposits in the mayer reagent test. Alkaloids are chemical compounds that are alkaline and contain a lot of nitrogen, have antibacterial properties, by inhibiting the formation of peptidoglycan on the bacterial cell wall, which causes instability of cell structure and leads to bacterial death. The second phytochemical test is the saponin test. The purpose of this test is to find out whether there is a content of saponin compounds by using aquadest and then shaken. Positive results in the saponin test are shown by the presence of foam formed on the surface. However, based on the results of the saponin test that has been carried out, bidara leaf extract, betel leaf, and a combination of negative results (-) do not form foam. This indicates that the extract does not contain saponins or that the content is so low that it is not qualitatively detectable. The third phytochemical test is the flavonoid test. The purpose of this test is to determine whether there is a content of flavonoid compounds contained in a part of the plant using Mg powder and concentrated HCl reagents. From the results of the flavonoid test carried out on positive (+) bidara leaf extract, a brick-orange red color is formed, a positive (+) betel leaf is formed an orange color and a positive (+) combination of brick-orange red is formed.

Flavonoids are known to have extensive biological activity, including as antibacterial, by forming complexes with bacterial cell wall proteins and disrupting the integrity of cell membranes. The fourth phytochemical test is the tannin test. The purpose of this test is to find out whether there is a content of tannin compounds contained in a part of the plant by using . Based on the results of the tannin test that has been carried out, in the extract of bidara leaves, betel leaves and a positive (+) combination of dark green color is formed. Tannins are phenolic compounds that can precipitate proteins and play a role in antibacterial activity by inactivating bacterial enzymes and depositing cell wall proteins, thereby inhibiting bacterial metabolism and growth. $FeCl_3$ The fifth phytochemical test is the terpenoid and steroid test. The purpose of this test is to determine whether there is a content of terpenoid compounds and steroids present in a part of the plant using the *Liebermann-Bouchard* method, the extract is dissolved in chloroform and then added to the *Liebermann-Bouchard reagent* (anhydrous acetate and concentrate). Based on the results of the terpenoid test that has been carried out, in the extract of bidara leaves, betel leaves and a combination of positive (+) brownish rings are formed. As for the results of steroid tests that have been carried out on bidara leaf extracts, betel leaves and a combination of negative results (-), bluish-green rings are not formed.

Terpenoid compounds are known to play a role in antibacterial activity by damaging the membrane structure and porine proteins on the bacterial cell wall thereby interfering with cell permeability. H_2SO_4 The sixth phytochemical test is the phenol test. The purpose of this test is to determine whether there is a content of phenol compounds contained in a part of the plant using NaCl and . Based on the results of the phenol test that has been carried out, in the extract of bidara leaves, betel leaves and a positive (+) combination of dark green color is formed. Phenol compounds have antibacterial activity by denatured proteins and damaging the cytoplasmic membrane of bacteria, leading to cell component leakage and lysis. $FeCl_3$ Based on the research that has been conducted, the results of the phytochemical test of bidara leaf extract obtained 5 secondary metabolic compounds, namely alkaloids, flavonoids, tannins, terpenoids and phenols. The results of previous research by Mochtar (2025) showed that the results of the phytochemical test of bidara leaves contained flavonoids of 12.8%, tannins 27.5%, saponins 89.8%, and phenols of 9.72%.

The results of another study by Ambrin *et al.*, (2024) obtained the results of a phytochemical test of bidara leaves that were positive for alkaloids, flavonoids, saponins, tannins, and terpenoids. Based on the research conducted, the results of the phytochemical test of betel leaf extract obtained 5 secondary metabolic compounds, namely alkaloids, flavonoids, tannins, terpenoids and phenols. The results of previous research by Sonphakdi *et al.*, (2024), obtained the results of phytochemical tests of betel leaves containing low alkaloids (+), low terpenoids (+), low saponins (*), high flavonoids (+++) and high tannins (+++). The results of previous research by Mariadi (2024) obtained the results of phytochemical tests on betel leaves containing positive flavonoids, saponins, tannins, and phenols. In the phytochemical test, the combination of bidara leaf and betel leaf extracts showed positive results including alkaloids, flavonoids, tannins, terpenoids, and phenols. These results show that the combination extract contains the same range of secondary metabolites as the single extract, but the phytochemical test is qualitative and cannot be used to quantitatively assess the increase in compound levels, so further quantitative analysis is needed to ascertain the difference in secondary metabolite levels.

Antibacterial Activity Test

This study aims to determine the antibacterial activity characterized by the formation of a clear barrier zone diameter around the disc paper in a combination of ethanol extract of bidara leaf (*Ziziphus mauritiana*) betel leaf (*Piper betle L*) with a concentration ratio of 100%:0%, 50%:50% and 0%:100% against the growth of *Staphylococcus aureus* bacteria using a sensitivity test with the disc method on MHA media (*Mueller-Hinton a*). The results showed that all treatment groups had antibacterial activity against *Staphylococcus aureus* characterized by the presence of clear zones in the area around the disc. The clear zone is the area that forms around the disc paper on the agar medium which indicates the absence of growth of microbial agents. The addition of antimicrobial agents to disc paper, will make the antimicrobial agent diffuse into the disc paper and inhibit the development of microbes around it. (Nurhayati *et al.*, 2020) The sensitivity of an active compound in inhibiting the growth of *Staphylococcus aureus* bacteria was evaluated based on the criteria of the *Clinical and Laboratory Standards Institute* (CLSI), divided into 3 categories,

namely sensitive (S), intermediate (I), and resistant (R). Based on the interpretation standards of antibiotic inhibition zones of the *Clinical and Laboratory Standards Institute (CLSI)*, (2024) *Clindamycin* is categorized as sensitive if it has an inhibitory zone diameter of ≥ 21 mm categorized as sensitive (S), 15–20 mm intermediate (I), and ≤ 14 mm resistant (R).

This is a reference to determine the interpretation of the inhibition zone of the combination of ethanol extract of bidara leaves (*Ziziphus mauritiana*) betel leaves (*Piper betle L*). The average diameter of the barrier zone that can be formed by the combination of ethanol extracts of bidara leaves (*Ziziphus mauritiana*) betel leaves (*Piper betle L*) in fighting *Staphylococcus aureus* bacteria shows a difference in each extract. In group 1 (100%:0%), the inhibition zone was recorded at 25.6 mm, group 2 (50%:50%) the inhibition zone was recorded at 19.4 mm and group 3 (0%:100%) the inhibition zone was recorded at 26.2 mm. Based on this data, the average results from groups 1 and 3 are included in the sensitive category (S) because the average yield of the inhibition zone produced is ≥ 21 mm. While group 2 is included in the intermediate category (I) because the average yield of the inhibition zone is 15–20 mm. Positive control indicates that the resulting inhibition zone diameter of 40 mm is in the sensitive category, and negative control with an inhibition zone diameter of 0 mm is in the resistant category. These results generally prove that ethanol extracts of bidara leaves and betel leaves, whether used alone or in combination, can inhibit the growth of *Staphylococcus aureus* bacteria. The positive control used in this study was the antibiotic *Clindamycin disc*, with a dose of 2 μ g per disc that was compliant with the CSLI standard.

The selection of *Clindamycin* as a positive control is because *Clindamycin* works against anaerobic bacteria, most gram-positive aerobic coccus bacteria, gram-positive and gram-negative bacilli, as well as some protozoa. *Staphylococcus aureus* bacteria are gram-positive bacteria (Saleh *et al.*, 2023). *Clindamycin* works by inhibiting the synthesis of bacterial proteins through binding to the 50S ribosomal subunit, thereby disrupting the peptide chain translocation process and inhibiting the growth of bacterial cells (Achyar, 2020). The negative control used in this study is 10% DMSO because the negative control must be in accordance with the solvent used as the diluent of the tested material (Hasanah & Rahayu, 2023). In this study, the negative control inhibition zone results using 10% DMSO against bacteria *Staphylococcus aureus* indicates a diameter of 0 mm or does not form an obstruction zone. Based on this, it can be concluded that 10% DMSO does not affect the test results. The use of negative control in this study was to compare that the solution used to dilute the test material did not affect the results of the antibacterial test. This is to show that the solution used as a test material diluent does not have antibacterial activity (Sangadji *et al.*, 2022). Comparison of group 1 (100%:0%) which contained only bidara leaf extract obtained an inhibition zone diameter of 25.6 mm with a sensitive category. Meanwhile, group 3 (0%:100%) which contained only betel leaf extract showed an inhibition zone diameter of 26.2 mm and was also classified as sensitive.

Although descriptively betel leaf extract produced a slightly larger inhibition zone diameter than bidara leaf extract, the results of the statistical test showed that there was no significant difference between the two groups ($p = 0.750$; $p > 0.05$). This indicates that bidara leaf and betel leaf extracts have antibacterial activity equivalent to *Staphylococcus aureus*. The small difference in numerical values is suspected to be influenced by biological and methodological factors. Biological factors include the genetic quality of the plant, the age of the leaves, and the growing environment conditions that affect the content and dominance of secondary metabolites. Methodological factors include extract concentration, test conditions, simplicia storage, extract storage time, and high water content in the extract, which directly affects antibacterial activity (Fitriana & Mukhlisah, 2024). Comparison of group 2 (50%:50%) which is a combination of 50% bidara leaf extract and 50% betel leaf, the diameter of the resulting inhibition zone is 19.4 mm and is categorized as intermediate. This value is descriptively lower than a single extract of 100% bidara leaves (25.6 mm) and a single extract of 100% betel leaves (26.2 mm). However, the results of the statistical test showed that there was no statistically significant difference in the diameter of the inhibition zone between the combination extract and a single extract of bidara leaves ($p = 0.075$) and betel leaves ($p = 0.116$). This shows that the addition of bidara leaf or betel leaf extract to the combination formulation has not provided an increase in antibacterial activity that can be statistically proven.

Thus, in the concentration and testing method used in this study, the combination of bidara leaf and betel leaf extract has not shown a significant synergistic effect compared to the use of a single extract. In addition to the concentration factor, variations in the diameter of the barrier zone between treatment groups were also influenced by biological and methodological factors. Biological factors include the genetic quality of the plant, the age of the leaves, and the growing environment conditions that affect the content and concentration of secondary metabolites. Methodological factors include test conditions, handling and storage of simplicia, duration of storage of extracts, and water content in extracts. Extracts with high water content and storage of either simplicia or extracts for a long time are susceptible to increased microorganism activity. The longer the storage time, the more microorganism growth will increase and trigger the decay process. The decay process causes changes in the chemical environment, one of which is an increase in pH value, which further supports the growth of microorganisms. This condition can result in damage to the extract, as microorganisms are able to degrade active compounds and produce toxic metabolites, so the antibacterial properties of the plant are reduced. (Fitriana & Mukhlisah, 2024). In addition, the interaction between secondary metabolite compounds in the combination of extracts also plays a role in determining the size of the inhibition.

Compound combinations do not always produce synergistic effects; interactions can be additive or even antagonistic, where one compound can inhibit the activity of another. The low diameter of the inhibition zone in the combination of bidara leaf and betel leaf extracts compared to single extracts indicates that the interactions of the compounds formed have not shown a synergistic effect, and are reinforced by the decrease in the effective levels of active compounds due to dilution. Based on the comparison of the results of this study, it can be stated that the combination of bidara leaf and betel leaf extract does have antibacterial activity against *Staphylococcus aureus*, so the hypothesis that the combination of the two has the ability to inhibit bacteria is acceptable. However, the data obtained did not show an increase in inhibition compared to single extracts of bidara and betel leaves. Thus, the combination of the two extracts does not provide a synergistic effect, but rather shows an additive effect or even slightly decreases the intensity of its antibacterial activity.

Therefore, although the combination of bidara leaf extract and betel leaf is still able to inhibit the growth of *Staphylococcus aureus*, its activity is not higher than the single extract used. Based on the results of phytochemical tests, the combination of bidara leaf and betel leaf extract contains various secondary metabolites that are known to have antibacterial potential, including alkaloids, flavonoids, tannins, terpenoids, and phenols, each of which acts through a different inhibitory mechanism against bacterial cells. Alkaloids work by inhibiting the formation of peptidoglycan on the bacterial cell wall, which causes instability of the cell structure and leads to bacterial death. Flavonoids have antibacterial activity by forming complexes with bacterial cell wall proteins and disrupting the integrity of cell membranes. Tannins work by inactivating bacterial enzymes as well as depositing cell wall proteins, thereby inhibiting bacterial metabolism and growth. Terpenoids act as antibacterials by damaging the membrane structure and porin proteins on the bacterial cell wall thereby interfering with cell permeability. Phenols are bactericidal by denaturing proteins and damaging the cytoplasmic membrane of bacteria, leading to cell component leakage and lysis (Sadiah *et al.*, 2022).

IV. CONCLUSION

The combination of ethanol extracts of bidara leaves (*Ziziphus mauritiana*) and betel leaves (*Piper betle* L.) against the growth of *Staphylococcus aureus* resulted in an average inhibition zone diameter in group 1 (100%:0%), 25.6 mm in the sensitive category, group 2 (50%:50%) 19.4 mm in the intermediate category and group 3 (0%:100%) 26.2 mm in the sensitive category. The combination of ethanol extracts of bidara leaf (*Ziziphus mauritiana*) and green betel leaf (*Piper betle* L.) had antibacterial activity against *Staphylococcus aureus*, but did not show synergistic effects and was no more effective than a single extract, as indicated by the diameter of the growth inhibition zone of *Staphylococcus aureus* in the in vitro disc diffusion method.

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