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Testing the Antibacterial Activity of Black Betel Leaf Extract (Piper betle L. Var Nigra) on Streptococcus mutans, Porphyromonas gingivalis, and Enterococcus faecalis

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Antibacterial, Black Betel Leaf Extract, Piper betle L. var nigra, Streptococcus mutans, Porphyromonas gingivalis, Enterococcus faecalis

Abstract
Black betel leaves (Piper betle L. var nigra) are known to have antibacterial effects caused by secondary metabolites such as tannins, phenolic compounds, saponins, flavonoids, alkaloids, and steroids. This study aims to assess the antibacterial activity of ethanol extract of black betel leaves (Piper betle L. var nigra) against S. mutans, P. gingivalis, and E. faecalis by measuring the diameter of the inhibition zone formed. This research is a type of pure experimental research (true experimental) with post test only control group design. This experimental research was conducted using the disc diffusion method in vitro, the bacteria were treated with black betel leaf extract (Piper betle L. var nigra) with a concentration variation of 60%, 70%, 80%, 90%, and 100%. The experiment was repeated three times. The results showed that black betel leaf (Piper betle L. var nigra) had antibacterial activity against two of the three bacteria. This activity was observed through the presence of distinct clear zones around the paper disks. In conclusion, extracts derived from black betel leaf (Piper betle L. var nigra) had antibacterial activity against S. mutans and P. gingivalis bacteria at 70%, 80%, 90%, and 100% concentrations. However, it did not have antibacterial activity against E. faecalis bacteria.

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INTRODUCTION

Health that needs attention apart from general body health is dental and oral health (Herawati et al., 2022) . If dental and oral health is disturbed, it will affect bodily health, thereby affecting the quality of human resources (Septiani, Sughesti, Susanti, Sihombing, & Novitasari, 2021) . The percentage of the population who have dental and oral problems according to RISKESDAS in 2007, 2013 and 2018 has always increased from 23.2% to 25.9% and in 2018 it became 57.6% (Herawati et al., 2022) . The prevalence of caries and periodontitis is very high in Indonesia, namely caries is in first position with a percentage of 88.8% and followed by periodontitis with a percentage of 74.1% (Riskesdas Team 2018, 2019).

The process of caries involves a number of factors that interact with each other, namely teeth and saliva (host), microorganisms, substrate and time (Ambarawati, Sukrama, & Yasa, 2020) . The role of microorganisms is very important in the process of dental caries which is also supported by other factors. Streptococcus mutans is a microorganism that causes dental caries which plays a major role in the onset of dental caries (Restina & Warganegara, 2016) . If caries is not treated immediately, dental treatment such as root canal treatment is necessary, however, in root canal treatment itself, root canal infections often occur. Endodontic treatment where primary infection or secondary infection occurs is usually caused by the colonization of microorganisms which are dominated by anaerobic bacteria, especially Enterococcus faecalis. (Nurbianti, Alhawaris, & Yani, 2021) .

Apart from caries, periodontal disease is also one of the dental and oral diseases that are often found in the world community, especially in Indonesia. (Setiawati, Robbihi, & Dewi, 2022) . Porphyromonas gingivalis is a gram-negative anaerobic bacterium that is involved in the pathogenesis of periodontitis (Ramadhani, Rudhanton, Diah, & Sutanti, 2022). Porphyromonas gingivalis is the most common bacteria associated with periodontitis, from subgingival plaque periodontitis patients, Porphyromonas gingivalis was found in 85.75% (Septiwidyati & Bachtiar, 2020) .

Current conditions in Indonesia show an increase in oral problems every year, including caries, periodontitis, and root canal infections which are a continuation of the caries process. This encourages health workers to make efforts to minimize the cause of these problems, namely oral bacteria. One of the efforts that can be made is to use mouthwash. Long-term use of mouthwash containing alcohol can cause dry mouth, reduce saliva production, and increase the risk of tooth decay and bad breath. Therefore, WHO recommends and promotes the use of traditional or herbal medicines in national health care programs because they are easily available, cheaper, and relatively safer without harmful side effects (Alamsyah, Arma, & Hidayati, 2021).

To obtain herbal products that are useful for treatment and prevention in the field of dentistry, many studies have been conducted to reduce the number of microorganisms in the oral cavity using natural ingredients. This is considered very beneficial because people have long believed that natural ingredients can treat various diseases with minimal side effects (Restina & Warganegara, 2016). In Indonesia, especially in tropical climates such as East Kalimantan, there are many biological plants that are utilized, one of which is the betel plant (Owu & Jayanti, 2020). Betel plants are known to be effective in controlling caries and periodontal disease (Agung, Hervina, & Sandi, 2021). Known betel varieties include green, red, and black betel (Zuraidassanaaz, 2017). Black betel leaves have antibacterial benefits (Saputri & Rahayu, 2018). Secondary metabolites of black betel leaf were identified to contain alkaloids, flavonoids, tannins, saponins, phenolic compounds, carotenoids, and steroids (Owu & Jayanti, 2020).

Black betel (Piper betle L. var nigra) is one type of betel that is widely found in Indonesia, but has not been widely recognized by the wider community (Qhorina, Prasetya, & Ardana, 2021). Research on betel leaf plants is quite a lot, but especially on black betel leaves (Piper betle L. var nigra) is still small. Prasetya (2012) conducted antimicrobial tests on black betel leaf extract and proved to be able to inhibit the growth of microbes Streptococcus mutans, Staphylococcus aureus, Candida albicans, and Candida utilis. Research by (Saputri & Rahayu, 2018) proved that 70% ethanol extract of black betel leaves has antibacterial activity against Staphylococcus aureus. Meanwhile, research by (Aprillia, Ardana, & Kuncoro, 2021) showed that ethanol extract of black betel leaves has antibacterial activity against the growth of Propionibacterium acne. Recent research by Herryawan et al. (2023) proved that black betel extract with concentrations of 25%, 50%, 75%, and 100% was effective in inhibiting the growth of Porphyromonas gingivalis bacteria with moderate to strong inhibition categories (Herryawan, Soerachman, & Chaeruddin, 2023).

This study aims to determine the antibacterial activity of black betel leaf extract (Piper betle L. var nigra) against the growth of the bacteria Streptococcus mutans, Porphyromonas gingivalis and Enterococcus faecalis. The specific aim is to determine the diameter of the inhibition zone of black betel leaf extract (Piper betle L. var nigra) against these three bacteria. The results of this research can be used to develop knowledge and scientific information in the field of dentistry, as input for health institutions to maximize the use of black betel leaves in health promotion activities, increase public information and knowledge about the potential of black betel leaves as an antibacterial agent, and provide practical and theoretical experience for researchers regarding the benefits of black betel leaves in inhibiting the growth of Streptococcus mutans, Porphyromonas gingivalis and Enterococcus faecalis bacteria.

RESEARCH METHODS

Research Design

This research is a type of pure experimental research (true experimental) with a post test only control group design. This experimental research was conducted using the disc diffusion method in vitro.

a Number of Treatments

This study used two different test groups, with several treatments:

The first group (test group) is a group consisting of five treatments on each of three bacteria namely Streptococcus mutans, Porphyromonas gingivalis and Enterococcus faecalis with the concentration of black betel leaf extract (Piper betle L. var nigra) which is 60%, 70%, 80%, 90% and 100%. The second group (control group) is positive control and negative control, positive control using Chlorhexidine gluconate 0.2%, negative control using DMSO 10%.

b Number of repetitions

This study was conducted three times (triplo) in the test group and also the control group.

Location and Time of Research

a Research Location

This research was conducted at the Pharmacology Laboratory of the Faculty of Medicine, Mulawarman University and UPTD. Health Laboratory of East Kalimantan Province.

b Research Time

The research time was conducted in February - May 2024.

Research Subjects and Sample Selection

a Test Bacteria Subjects

The test bacterial subjects used in this study were Streptococcus mutans, Porphyromonas gingivalis, and Enterococcus faecalis obtained from UPTD. Samarinda City Provincial Health Laboratory.

b Test Plant Subjects

The plant subjects used in this study were black betel leaves (Piper betle L. var nigra) purchased at Jalan Mugirejo, Samarinda City, East Kalimantan. Identification of plant subjects was carried out by the Faculty of Forestry, Mulawarman University.

Research Tools and Materials

a Research Tools

The tools used in this study are stationery, handscoon, sterile masks, digital scales, drying cabinets, blenders, maceration containers, macerate containers, vials, erlenmeyer flasks, hot plate stirrers, petridish, autoclave, rotary evaporator (rotav), test tubes, test tube racks, micropipettes, paper disks, analytical scales, sterile disposable ose, sterile cotton swab, vortex mixer, nephelometer, tweezers, anaerobic jar, incubator, vernier, Biological Safety Cabinet (BSC).

b Research Materials

The materials used in this study were black betel leaves (Piper betle L var. nigra), sterile distilled water, 70% ethanol, Nutrient Agar (NA), Mueller Hinton Agar (MHA), 5% sheep blood, Blood Agar Base (blood agar), sterile NaCl 0.9%, Chlorhexidine gluconate 0.2%, DMSO 100%, Streptococcus mutans, Porphyromonas gingivalis and Enterococcus faecalis bacteria.

Research Variables

a The dependent variable

The dependent variable in this study is the inhibition of three oral bacteria, namely Streptococcus mutans, Porphyromonas gingivalis and Enterococcus faecalis.

b Free Variable

The independent variable in this study is the concentration of black betel leaf extract (Piper betle L var. nigra).

c Control variable

The control variables in this study consisted of two, namely positive control and negative control. Positive control is a treatment that produces an effect on the dependent variable. This study used Chlorhexidine gluconate 0.2% as a positive control. Chlorhexidine gluconate 0.2% is a broad-spectrum antimicrobial agent that is one of the gold standards for plaque prevention in dentistry. Chlorhexidine gluconate 0.2% has a bacteriostatic effect and a bactericidal effect against all types of microbes, both gram-positive and gram-negative bacteria. Negative control is a treatment that does not produce an effect on the dependent variable. This study used 10% Dimethyl Sulfoxide (DMSO) as a negative control because DMSO has no effect on bacterial inhibitory activity and is generally only used as a solvent.

RESULTS AND DISCUSSION

Antibacterial Activity Test Results Streptococcus mutans

The antibacterial activity test was carried out using five concentrations of black betel leaf extract, namely concentrations of 60%, 70%, 80%, 90% and 100% and using a positive control of 0.2% Chlorhexidin gluconate and a negative control of 10% DMSO where the test group and control group were repeated three times.

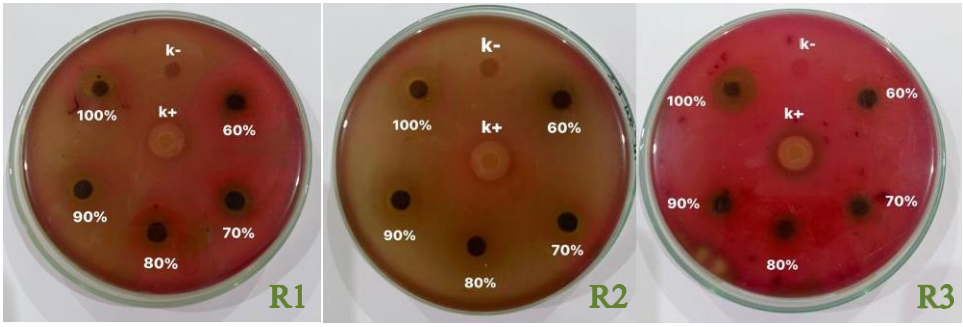

Figure 1 Results of antibacterial activity test for treatment groups against Streptococcus mutans bacteria.
Source: Primary Data

The results of the research were seen by measuring the diameter of the inhibition zone formed around the paper discs 24 hours after being treated using a vernier caliper in millimeters (mm) and then making a table, which then included the data. The measurement results are processed and analyzed using the SPSS software application.

Table 1 Antibacterial Activity Test Results for Treatment Groups against Streptococcus mutans

Treatment group	Diameter zone resistor (mm)			Average (mm) ±SE	*One Way ANOVA test*
	R1	R2	R3		
60%	0	0	0	0.00±0.000	
70%	1.00	0.52	0	0.50±0.288	
80%	1.1	1.56	0	0.88±0.462	
90%	2.05	2.58	0	1.54 ± 0.786	0,000
100%	2.88	2.00	0	1.62±0.852	
CHX 0.2 (K +)	6.22	6.48	5.38	6.02±0.331	
DMSO 10% (K -)	0	0	0	-	

Information: R = Repetition (repetition); - = cannot inhibit

Table 1 presents the results of the average diameter of the inhibition zone (mm) for Streptococcus mutans obtained from treatment with each extract concentration in three repetitions where these results have been reduced by the diameter of the paper disc (6 mm). The results of treatment of the test groups respectively at concentrations of 60%, 70%, 80%, 90% and 100% showed an average diameter of the inhibition zone of 0.00 ± 0.000 mm, 0.50 ± 0.288 mm, 0.88 ± 0.462 mm, 1.54 ± 0.786 mm , and 1.62 ± 0.852 mm. Meanwhile, the results of the positive control treatment, namely CHX 0.2 or Chlorhexidine gluconate 0.2%, had an average inhibitory zone diameter of 6.02 ± 0.331 mm, and the negative control DMSO 10% did not produce a clear zone or an inhibitory zone equal to 0 mm.

The Shapiro-Wilk normality test (Appendix 8) shows that all treatments have a significance value of p>0.05, while the Lavene's homogeneity test has a significance value of p<0.05, so in this case the research data can be said to be normally distributed and not homogeneous, but to test Differences between several treatment groups can still be used using the One-Way ANOVA Test. The p-value in the Sig column. shows a value of 0.000, where the value of 0.000 < 0.05 which means there is a significant difference in the treatment group, to find out which group has a significant difference, it is carried out Post-Hoc test analysis (Appendix 8). Because the variants used are not the same, the analysis used is the Games-Howell test.

Table 2 Post-hoc test results (Games Howell)

Games Howell	60%	70%	80%	90%	100%	K+
60%		,610	,556	,542	,558	.011*
70%	,610		,971	,802	,799	.002*
80%	,556	,971		,967	,958	.007*
90%	,542	,802	,967		1,000	,075
100%	,558	,799	,958	1,000		,097
K+	.011	,002	,007	,075	,097	

Note: * = has a significant difference (p<0.05)

In Table 2 and also in Appendix 8, the results of the Post-Hoc test on Streptococcus mutans bacteria are presented, where the Sig value. The positive control had a significant difference with concentrations of 60%, 70% and 80% (p<0.05). This shows that Chlorhexidine gluconate 0.2% is better at inhibiting Streptococcus mutans bacteria than this concentration. However, at concentrations of 90% and 100% there was no significant difference (p>0.05) with the positive control, so it can be said that its antibacterial power is almost as strong as Chlorhexidine gluconate 0.2%.

Porphyromonas gingivalis

The antibacterial activity test was carried out using five concentrations of black betel leaf extract, namely concentrations of 60%, 70%, 80%, 90% and 100% and using a positive control of 0.2% Chlorhexidin gluconate and a negative control of 10% DMSO where the test group and control group were repeated three times.

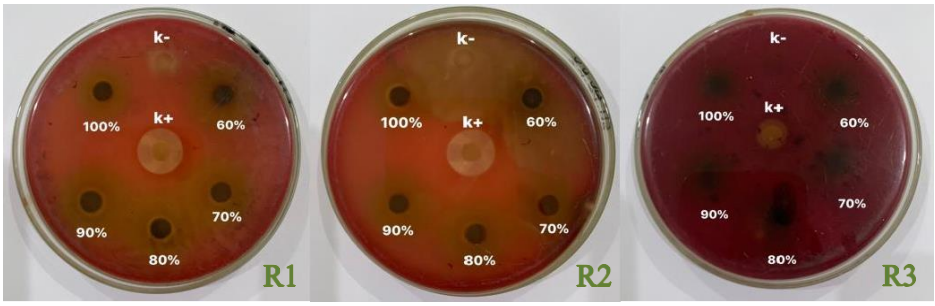

Figure 2 Results of antibacterial activity test for treatment groups against Porphyromonas gingivalis bacteria.
Source: Primary Data

The results of the research were seen by measuring the diameter of the inhibition zone formed around the paper discs 24 hours after being treated using a vernier caliper in millimeters (mm) and then making a table, and then the measurement data was processed and analyzed using the SPSS software application.

Table 3 Antibacterial Activity Test Results for Treatment Groups against Porphyromonas gingivalis

Treatment group	Diameter zone resistor (mm)			Average (mm) ±SE	*Kruskal Wallis test*
	R1	R2	R3		
60%	0	0.3	0	0.10±0.100	0.079
70%	0.68	0.65	0	0.44±0.221	
80%	1.4	1.4	0	0.93±0.466	
90%	2.1	1.6	0	1.23±0.633	
100%	2.78	2.62	0	1.80±0.901	
CHX 0.2 (K +)	8.28	8.35	3.75	6.79±1.521	
DMSO 10% (K -)	0	0	0	-	

Information: R = Repetition (repetition); - = cannot inhibit

Table 3 presents the results of the average diameter of the inhibition zone (mm) against Porphyromonas gingivalis which obtained from behavior each extract concentration in three repetitions where the results were reduced by the diameter of the paper disc (6 mm). The results of the treatment of the test groups respectively at concentrations of 60%, 70%, 80%, 90% and 100% showed an average diameter of the inhibition zone of 0.10 ± 0.100 mm, 0.44 ± 0.221 mm, 0.93 ± 0.466 mm, 1.23 ± 0.633 mm , and 1.80 ± 0.901 mm. Meanwhile, the results of the positive control treatment, namely CHX 0.2 or Chlorhexidin gluconate 0.2%, had an average inhibitory zone diameter of 6.79 ± 1,521 mm, and the negative control DMSO 10% did not produce a clear zone or an inhibitory zone equal to 0 mm.

The Shapiro-Wilk normality test (Appendix 9) shows that several treatments have a significance value of $p<0.05$, and the Lavene's homogeneity test has a significance value of $p<0.05$, so in this case the research data can be said to be not normally distributed and not homogeneous, so that To test differences between several groups using an alternative test, namely the Kruskal Wallis Test. The p-value in the Asymp column. Sig. shows a value of 0.079, where 0.079 > 0.05, which means there is no significant difference in each treatment group. This shows that each extract concentration with the positive control is almost equivalent in its antibacterial strength.

Enterococcus faecalis

The antibacterial activity test was carried out using five concentrations of black betel leaf extract, namely concentrations of 60%, 70%, 80%, 90% and 100% and using a positive control of 0.2% Chlorhexidin gluconate and a negative control of 10% DMSO where the test group and control group were repeated three times.

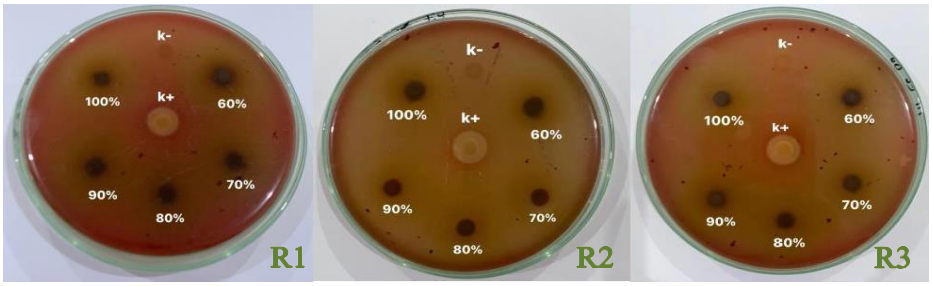

Figure 3 Results of antibacterial activity test for treatment groups against Enterococcus faecalis bacteria.
Source: Primary Data

The results of the research were seen by measuring the diameter of the inhibition zone formed around the paper discs 24 hours after being treated using a vernier caliper in millimeters (mm) and then making a table, and then the measurement data was processed and analyzed using the SPSS software application.

Table 4 Antibacterial Activity Test Results for Treatment Groups against Enterococcus faecalis

Treatment Group	Diameter zone resistor (mm)			Average (mm) ±SE	*Kruskal Wallis test*
	R1	R2	R3		
60%	0	0	0	0.00±0.000	
70%	0	0	0	0.00±0.000	
80%	0	0	0	0.00±0.000	
90%	0	0	0	0.00±0.000	0.005
100%	0	0	0	0.00±0.000	
CHX 0.2 (K +)	4.88	5.68	5.05	5.20±0.243	
DMSO 10% (K -)	0	0	0	0.00±0.000	

Information: R = Repetition (repetition); - = cannot inhibit

Table 4 presents the results of the average diameter of the inhibition zone (mm) against Enterococcus faecalis obtained from the treatment of each concentration extract in three repetitions where the results have been reduced by the diameter of the paper disc (6mm). The results of the treatment of the test group at all concentrations, namely 60%, 70%, 80%, 90% and 100%, showed an average diameter of the inhibition zone of 0.00 ± 0.000 mm, which means there was no inhibition zone at all. Meanwhile, the results of the positive control treatment, namely CHX 0.2 or Chlorhexidine gluconate 0.2%, had an average diameter of the inhibition zone of 6.79 ± 1.521 mm, and the negative control 10% DMSO did not produce a clear zone or the zone of inhibition was equal to 0 mm.

The Shapiro-Wilk normality test and the Lavene's homogeneity test (Appendix 10) both show that the treatment group has a significance value of $p<0.05$, so in this case the research data can be said to be not normally distributed and not homogeneous, so as to test the differences between several groups using an alternative test, namely the Kruskal Wallis Test. The p-value in the Asymp column. Sig. shows a value of 0.005, where the value 0.005 <0.05, which means there is a significant difference in the treatment group. This difference can be seen from (Appendix 10) visualization dun test, that the inhibition zone of the positive control Chlorhexidine gluconate 0.2% has a significant difference to all test groups.

The test plant used in this research was the black betel leaf plant (Piper betle L. var nigra). Black betel leaves were determined to ensure that the plants used were truly black betel leaves (Piper betle L. var nigra). Determination was carried out at the Tropical Forest Ecology and Biodiversity Conservation Laboratory, Faculty of Forestry, Mulawarman University. Antibacterial activity testing in this study was carried out using the disc diffusion method to see the diameter of the inhibition zone. In this study, three bacteria were used, namely Streptococcus mutans ATCC 35668, Porphyromonas gingivalis and Enterococcus faecalis ATCC 29212. Preparation of black betel leaf simplicia powder was carried out at the Pharmacology Laboratory, Faculty of Medicine, Mulawarman University. The antibacterial activity test was carried out at the East Kalimantan Provincial Health Laboratory UPTD using Bio Safety Cabinet Class II (BSC II) to prevent contamination.

Interpretation and Discussion of Results

The results of this research prove that black betel leaf extract (Piper betle L. var nigra) can inhibit two of the three bacteria tested, namely Streptococcus mutans and Porphyromonas gingivalis, as indicated by the presence of a clear zone or inhibition zone that is formed. This inhibition zone is evidence that there is antibacterial activity in the sample of black betel leaf extract (Piper betle L. var nigra) used by researchers. Antibacterial

activity can be influenced by several factors which are divided into biological factors and technical factors. Technical factors can mostly be controlled by researchers but biological factors cannot be controlled by researchers. Technical factors that influence the size of the inhibition zone in the disc diffusion method include: inoculum concentration, disc installation time, incubation temperature, incubation time, plate size, agar media thickness and media composition. (Nor, Indriarini, & Koamesah, 2018) . The turbidity of the bacterial suspension used has been adjusted to the McFarland standard of 0.5 or the equivalent of 1x108 bacteria/mL which has been confirmed using a nephelometer (Aprillia et al., 2021) . The medium used to test antibacterial activity on the bacteria Streptococcus mutans and Enterococcus faecalis is Mueller Hinton Agar + 5% sheep blood which has been adapted to the latest CLSI standards. Meanwhile, the test medium for Porphyromonas gingivalis bacteria is Blood Agar, which is adapted to previous research, namely (Nabila, Purnamasari, & Alhawaris, 2021) which also used the disc diffusion method for Porphyromonas gingivalis bacteria. The temperature and incubation time in this study have been adjusted to 1x24 hours with a temperature of 35 ± 20C (CLSI, 2020; Hudzicki, 2016). Meanwhile, biological factors consist of persisters and resistance. Persisters come from cells that are dormant or replicate slowly so they cannot be killed by antibacterial agents. The persister's factor has been controlled by using an inoculum that does not exceed 24 hours or an inoculum in the logarithmic phase. Resistance cannot be controlled in research because it is an adaptation of bacteria to survive (MOJA, 2015) .

Antibacterial activity is also influenced by several factors such as the content of antibacterial compounds, extract concentration, extract diffusion power, and the type of bacteria being inhibited (Fitriani, 2014) . The content of antibacterial compounds extracted from a plant is influenced by the solvent so that the choice of solvent is important in the diffusion power of the extract (MOJA, 2015) . The choice of solvent is based on its ability to have large polarity or be semi polar so that it can dissolve various chemical components in samples that are polar to nonpolar in maximum amounts (Handoyo, 2020) . The solvent chosen for this research was 70% ethanol solvent, because the antibacterial compounds thought to be found in black betel leaves (Piper betle L. var nigra) include tannins, phenolic compounds, saponins, flavonoids, alkaloids, steroids (Prasetya & Angga, 2013) , and terpenoids (Maharani & Fernandes, 2021) . These compounds are polar, semi-polar and non-polar compounds (steroids and terpenoids). Polar compounds will dissolve in polar filter solutions and nonpolar compounds will dissolve or disperse in nonpolar solvents. 70% ethanol is more polar than 96% ethanol and more non-polar than 50% ethanol, so compounds that are polar and non-polar will tend to dissolve more in 70% ethanol. The choice of 70% ethanol solvent is expected to optimize the content of the compounds contained in the extract so that it has antibacterial power (Kamaruddin & Arnov, 2023) . Several studies that also used 70% ethanol solvent to extract antibacterial compounds in black betel leaves (Piper betle L. var nigra) are (Aprillia et al., 2021) .

Compounds that may play a role in causing the antibacterial activity of black betel leaf extract (Piper betle L. var nigra) are active compounds in the phenol group and their derivatives, especially tannins and flavonoids (Owu & Jayanti, 2020) . Phenolic compounds are polar and act as antibacterials. The mechanism of action of phenol compounds in killing bacterial cells is by denaturing bacterial cell proteins. As a result of the denaturation of bacterial cell proteins, all bacterial cell metabolic activities stop because all bacterial cell metabolic activities are catalysed by enzymes which are proteins. At high concentrations, phenol content can penetrate and disrupt bacterial cell walls and precipitate proteins in bacterial cells, while at lower concentrations phenol inactivates important enzyme systems in bacterial cells (Marfuah, Dewi, & Rianingsih, 2018) .

Tannin is a derivative of phenol compounds. Tannins have antibacterial activity by precipitating proteins, inactivating enzymes, and destroying or inactivating genetic material. The antibacterial properties of tannins depend on their chemical structure and molecular weight. Low molecular weight tannins have better activity than higher molecular weight tannins (Kurniasari, 2022) . Tannins can be classified into condensation tannins and hydrolysis tannins. Previous research showed that only hydrolysed tannin showed

antibacterial activity, hydrolysed tannin was found to have much better antibacterial activity than condensed tannin or a mixture of the two (Fitriani, 2014) .

Flavonoids are the largest group of phenolic compounds (Marfuah et al., 2018) . Flavonoids are antibacterial by binding to proteins via hydrogen bonds, causing the protein structure to be damaged. Most cell wall structures and cytoplasmic membranes contain proteins and fats. Flavonoids also break the bonds between N-Acetyl glucosamine and N-Acetylramic acid which is found in the peptidoglycan layer of cell membranes. Damage to the peptidoglycan layer which is the framework of the cell membrane will result in instability of the cell membrane and the bacterial wall, causing the selective permeability function, active transport function, control of the protein structure of the bacterial cell to be disturbed which will result in the escape of macromolecules and ions from the cell, so that the bacterial cell becomes loses shape and lysis occurs (Ernita Sari, Rahmawan, & Sahara, 2021) . Phenol is able to cause coagulation of cell proteins and lyse cells at high levels, whereas at low concentrations phenol protein complexes are formed with weak bonds and immediately decompose so that the antibacterial effect becomes weak (Fitriani, 2014) .

Other compounds which are also thought to act as antibacterial include saponins, alkaloids, steroids and terpenoids. Saponins, which are detergents, have amphipathic molecules (containing hydrophilic and hydrophobic parts) which can dissolve membrane proteins. The hydrophobic end of saponin binds to the hydrophobic region of cell membrane proteins by displacing some of the bound lipid elements, resulting in bacterial cell lysis. The mechanism of action of alkaloids as antibacterial is by disrupting the peptidoglycan components in bacterial cells so that the cell wall layer does not form completely and causes cell death. Apart from that, alkaloids also inhibit the formation of protein synthesis so that they can disrupt bacterial metabolism (Fajrina, Bakhtra, Eriadi, Putri, & Wahyuni, 2021) .

Steroids have antibacterial activity through interaction with cell phospholipid membranes which are permeable to lipophilic compounds, causing membrane integrity to decrease and cell membrane morphology to change which can cause bacterial cells to become brittle and lyse (Saputri & Rahayu, 2018) . Meanwhile, terpenoid compounds have antibacterial activity through reactions with porins or Tran's membrane proteins in the outer membrane of bacterial cell walls, forming strong polymer bonds, resulting in the destruction of poring. Damage to poring, which is the gateway for compounds to enter and exit, will reduce the permeability of bacterial cell walls. This cell wall permeability will disrupt the transport of nutrients and other compounds, so that bacterial growth is hampered or dies (Ernita Sari et al., 2021) . Flavonoids, tannins, saponins and steroids are known to work synergistically in inhibiting bacterial growth.

Antibacterial Activity of Black Betel Leaf Extract Against Streptococcus mutans Bacteria

The results of research testing the antibacterial activity of black betel leaf extract (Piper betle L. var nigra) were proven to inhibit the growth of Streptococcus mutans by forming a clear zone or the diameter of the inhibitory zone around the paper disc. This research is in accordance with research conducted by previous researchers that betel leaves can inhibit the growth of gram-positive bacteria, namely Streptococcus mutans bacteria (Owu & Jayanti, 2020) . This is because gram-positive bacteria only have a single layer in their cell walls, little lipids and lots of echoic acids. Echoic acid is a polymer that is soluble in water and is polar. Most of the antibacterial compounds in black betel leaves are polar compounds, so these compounds can easily penetrate the peptidoglycan layer of gram-positive bacteria which are also polar. (Magvirah, Marwati, & Ardhani, 2020) .

The results of the treatment in the test group, precisely at a concentration of 60%, did not show a clear zone forming around the disc, indicating that at this concentration black betel leaf extract (Piper betle L. var nigra) could not inhibit Streptococcus mutans bacteria at all. Meanwhile, at concentrations of 70%, 80%, 90% and 100%, a clear zone can be seen forming around the disc and has an average diameter of the inhibition zone respectively 0.50 ± 0.288 mm, 0.88 ± 0.462 mm, 1.54 ± 0.786 mm, and 1.62 ± 0.852 mm which shows that at this concentration black betel leaf extract (Piper betle L. var nigra) can inhibit Streptococcus

mutans bacteria. Then the results were interpreted based on the categories of Davis & Stout (1971), and it was found that black betel leaf extract (Piper betle L. var nigra) at concentrations of 70%, 80%, 90% and 100% had weak strength in inhibiting the growth of Streptococcus bacteria. mutants.

The results of this research are different from previous research conducted by Ningsih in 2013, she proved that red betel leaf extract (Piper crocatum) at a concentration of 100% had an inhibitory zone diameter of 11.78 mm which was classified as strong. This can occur due to differences in the types of betel leaves used, where similar plants may contain the same secondary metabolites but in different quantities (Qhorina et al., 2021) . In accordance with Hasanah's research in 2020, red betel leaf extract (Piper crocatum) has higher antibacterial effectiveness in inhibiting bacterial growth, because there are active compounds in red betel leaves (Piper crocatum) which are stronger than black betel leaves (Piper betle L). . var nigra). Apart from the type of plant, different results can occur due to differences in the test methods used. In Ningsih's (2013) research, he used the well diffusion method, where the antibacterial activity using the well method had a larger zone compared to the disc method. This is thought to be because the isolate is active not only on the surface of the agar but also down to the bottom, resulting in a more homogeneous and efficient osmosis process in inhibiting bacterial growth (Nurhayati, Yahdiyani, & Hidayatulloh, 2020) . The method in this research, namely disc diffusion, was chosen because it is practical, easy to do, does not require special equipment and is relatively cheap. However, the size of the inhibition zone formed depends on the incubation conditions, inoculum, prediffusion and preincubation as well as the thickness of the medium (Rizki, Latief, Fitrianingsih, & Rahman, 2022) .

In Appendix 8, multiple comparisons of Streptococcus mutans bacteria show that the positive control inhibition zone has a significant difference with concentrations of 60%, 70%, 80% ($p<0.05$). This shows that Chlorhexidine gluconate 0.2% is better at inhibiting Streptococcus mutans bacteria than this concentration. However, at concentrations of 90% and 100% there was no significant difference ($p>0.05$) with the positive control, so it could be said that its antibacterial power was almost equivalent to Chlorhexidine gluconate 0.2%. The positive control in this study, namely Chlorhexidine gluconate 0.2%, had an average inhibition zone diameter of 6.02 ± 0.331 mm. Chlorhexidine gluconate 0.2% was chosen as a positive control because this compound has significant antibacterial ability against gram-positive bacteria, one of which is Streptococcus mutans, in accordance with the test bacteria in this study (Pambudi, Wasiaturrahmah, & Aspriyanto, 2021) . Chlorhexidine contains phenol which has a bacteriostatic effect at levels of 0.2-1%, is bactericidal at levels of 0.4-1.6%, and is fungicidal at levels above 1.3%. The basic ingredient chlorine is a high level disinfectant because it is very active on all bacteria, viruses, fungi, parasites and some spores. (Pradayani, Pertiwi, & Ambarawati, 2021) . The mechanism of action is to disrupt the cell membrane transportation process and bacterial metabolism, so that the cell wall becomes lysed. The process begins with the 0.2% Chlorhexidine gluconate compound binding the Streptococcus mutans bacteria, which is caused by ionic bonds in the form of attracting cations from the Chlorhexidine molecule and anions of the Streptococcus mutans cell wall. This ionic bond will cause a selective increase in the permeable peptidoglycan of Streptococcus mutans so that the cell membrane becomes damaged, the cytoplasm leaks, ultimately causing the death of the bacteria (Pambudi et al., 2021) .

The negative control used in this study, namely 10% DMSO, did not show any inhibition zone at all, which proves that 10% DMSO as an extract solvent does not have antibacterial activity and does not affect the test results in this study. DMSO was chosen because it can dissolve almost all compounds, both polar and non-polar. DMSO is a compound that has low toxicity, has anti-inflammatory and analgesic effects (Rahmi & Putri, 2020) . DMSO has the ability to penetrate cell membranes, however, when using DMSO as a solvent, the final concentration of DMSO should not exceed 10% because it can cause cell membrane rupture. The 10% DMSO solvent is an organic solvent and is not bactericidal so it cannot affect the test results (Manarisip, Fatimawa, & Rotinsulu, 2020) .

The results of this research also show that each increase in the concentration of black betel leaf extract (Piper betle L. var nigra) given will result in an increasingly larger diameter of the inhibition zone. This research is in accordance with Owu's research in 2020 which used betel leaf extract (Piper betle L) against Streptococcus mutans bacteria and Saputri & Rahayu's research in 2018 which used black betel leaf extract (Piper betle L. var nigra) against Staphylococcus aureus bacteria (Owu & Jayanti, 2020) . This research also shows that the diameter of the inhibition zone is directly proportional to the concentration of betel leaf extract. The greater the concentration, the greater the active substance dissolved, because the less diluent is used. So a solution with a higher concentration will have a more concentrated active substance (Amanda, Mastra, & Sudarmanto, 2018) .

Antibacterial Activity of Black Betel Leaf Extract Against Porphyromonas gingivalis Bacteria

The results of the antibacterial activity test of black betel leaf extract (Piper betle L. var nigra) were proven to inhibit the growth of Porphyromonas gingivalis by forming a clear zone or the diameter of the inhibitory zone around the paper disc. This research is in accordance with research conducted by Herryawan in 2023 & Sendy in 2014 that green, red and black betel leaves can inhibit the growth of gram-negative bacteria, namely Porphyromonas gingivalis bacteria (Herryawan et al., 2023) . This can happen because gram-negative bacteria have thin peptidoglycan cell walls, only about 10% of the dry mass of their cell walls. Gram-negative bacteria contain a lot of lipids and have porin proteins which act as channels for the entry of active substances into bacterial cells. The entry of this active substance damages enzyme activity in cells and causes cell damage. High lipid levels in cells will increase the permeability of active substances into cells (Rompas, Wewengkang, & Mpila, 2022) . One of the compounds that is thought to play an important role in these bacteria is steroids, where steroids can interact with cell phospholipid membranes which are permeable to lipophilic compounds, causing membrane integrity to decrease and cell membrane morphology to change causing cells to become brittle and lysed (Nisa, 2019).

The results of treatment at concentrations of 60%, 70%, 80%, 90% and 100% showed the average diameter of the inhibition zone respectively, namely 0.10 ± 0.100 mm, 0.44 ± 0.221 mm, 0.93 ± 0.466 mm, 1.23 ± 0.633 mm, and 1.80±0.901 mm. These results were interpreted based on the categories of Davis & Stout (1971), and it was found that black betel leaf extract (Piper betle L. var nigra) at all concentrations had weak power in inhibiting the growth of Porphyromonas gingivalis bacteria.

The results of this research are slightly different from research conducted by Herryawan in 2023, where he proved that black betel leaf extract (Piper betle L. var nigra) based on the category of David & Stout (1971), at a concentration of 100% is classified as strong (mean: 19.65mm) in inhibiting the growth of Porphyromonas gingivalis bacteria (Herryawan et al., 2023) . This is thought to occur due to many factors, including differences in the origin of the plant and the extraction process, considering the different geographical conditions and climate changes which result in variations in the chemical compound content of a plant. The black betel leaf plant (Piper betle L. var nigra) used in this research comes from Samarinda, which is planted on vines on tree stands as a natural stake in the yard, protected by shade, without fertilizer application since planting and aged at harvest ± 3 years. Where these factors can influence the quality and content of black betel leaves (Piper betle L. var nigra) produced, so that they can cause different results from other studies even though they use the same type of plant. In the book "Cultivation and benefits of betel for health" by Widiyastuti, Rahmawati and Mujahid (2020), it is stated that in conditions under shade, with light intensity around 50%, betel is still able to grow with morphological changes in the form of wider and thinner leaves. However, betel will produce better quality leaves if planted in an open area with full sunlight. Harvesting betel must also be done at the right age and time (1-2 years after planting) to obtain the most optimal quality. Planting betel in less fertile soil requires additional (organic) fertilizer so that the soil in which it grows can better hold water.

In Appendix 9, the results of the Kruskal Wallis test on Porphyromonas gingivalis bacteria, the P-value in the Asymp column. Sig. shows a value of 0.079, where 0.079 > 0.05, which means there is no significant difference in each treatment group. This shows that each treatment in this study has an inhibition zone that is not significantly different or is almost equivalent in its antibacterial strength against Porphyromonas gingivalis bacteria. This shows that the positive control used, namely Chlorhexidin gluconate 0.2%, is almost equivalent in antibacterial strength to the entire concentration of black betel leaf extract (Piper betle L. var nigra) in inhibiting the growth of Porphyromonas gingivalis bacteria.

The positive control in this study, namely Chlorhexidine gluconate 0.2%, had an average inhibition zone diameter of 6.79 ± 1,521 mm. Chlorhexidine gluconate 0.2% was chosen as a positive control because it is the gold standard mouthwash which has broad spectrum antimicrobial properties and is effective against various types of gram-positive, gram-negative bacteria and fungi, and is a mouthwash that is often used in chronic periodontitis therapy. Chlorhexidine molecules have a positive charge (cation) and most bacterial molecular charges are negative (anions). This causes strong attachment of Chlorhexidine to the bacterial cell membrane. Chlorhexidine will cause changes in the permeability of bacterial cell membranes, causing the release of cell cytoplasm and low molecular weight cell components from inside the cell through the cell membrane, causing bacterial death.

The negative control used in this study, namely 10% DMSO, did not show any inhibition zone at all, which proves that 10% DMSO as an extract solvent does not have antibacterial activity and does not affect the test results in this study. DMSO was chosen because it can dissolve almost all compounds, both polar and non-polar. DMSO is also a compound that has low toxicity, has anti-inflammatory and analgesic effects (Rahmi & Putri, 2020) . DMSO has the ability to penetrate cell membranes, however, when using DMSO as a solvent, the final concentration of DMSO should not exceed 10% because it can cause cell membrane rupture. The 10% DMSO solvent is an organic solvent and is not bactericidal so it cannot affect the test results (Manarisip et al., 2020) .

The results of this research also show that each increase in the concentration of black betel leaf extract (Piper betle L. var nigra) given will result in an increasingly larger diameter of the inhibition zone. This research is in accordance with Herryawan's research in 2023, which used extracts of red betel leaves (Piper crocatum) and black betel leaves (Piper betle L. var nigra) against Porphyromonas gingivalis bacteria (Herryawan et al., 2023) . Supported by Sendy's research in 2014, which used red betel leaf extract (Piper crocatum) against Porphyromonas gingivalis bacteria. This research also shows that the diameter of the inhibition zone is directly proportional to the concentration of betel leaf extract. The greater the concentration, the greater the active substance dissolved, because the less diluent is used. So a solution with a higher concentration will have a more concentrated active substance (Amanda et al., 2018) .

Antibacterial Activity of Black Betel Leaf Extract Against Enterococcus faecalis Bacteria

The results of the antibacterial activity test of black betel leaf extract (Piper betle L. var nigra) in this study proved that it could not inhibit the growth of Enterococcus faecalis bacteria. This research is in accordance with research conducted by Wastri in 2021 that basil leaf extract (O. basilicum) has no inhibition zone against Enterococcus faecalis and also Wijaya's research in 2021 that there is no antibacterial effectiveness of Virgin Coconut Oil (VCO) extract against Enterococcus faecalis. In 2009, Limsuwan also carried out extensive screening of 31 plant species with 12 pathogenic bacteria, and proved that betel leaves (Piper betle) showed significant activity against almost all species, except for one bacteria, namely Enterococcus faecalis.

The results of this research are different from the results of Armianty & Mattulada's research in 2014 which proved that betel leaf extract could inhibit the growth of Enterococcus faecalis bacteria at a concentration of 20%, as well as Pasril & Yuliasant's research in 2022 which proved that red betel leaf extract (Piper crocatum) able to inhibit the

growth of Enterococcus faecalis bacteria. This can occur due to differences in the types of betel leaves used, where similar plants may contain the same secondary metabolites but in different quantities (Qhorina et al., 2021) . This is also in accordance with Hasanah's research in 2020 that red betel leaf extract (Piper crocatum) has higher antibacterial effectiveness in inhibiting bacterial growth, because there are active compounds in red betel leaves (Piper crocatum) which are stronger than black betel leaves (Piper betle L. var nigra). Each type of betel leaf has a different quantity of secondary metabolite content, so that at the same concentration you can get different results. The content of secondary metabolites in a plant is influenced by several factors, both internal and external. Internal factors such as genes and external factors include light, temperature, humidity, pH, nutrient content in the soil and altitude. Different altitudes will produce different temperatures. A series of metabolic processes in plants will be disrupted so that the compounds produced from these processes will be different at each altitude. This can affect the amount of antibacterial substances contained in the sample and can cause the inhibition zone not to form due to its inability to damage cell membranes and disrupt cell physiological processes (Nabila et al., 2021) .

Apart from the type of betel leaf used, this difference could be caused by the bacteria being inhibited, where the bacteria used in this study were pure culture bacteria obtained from the Regional Health Laboratory and it cannot be known how many times the bacteria have been cultured. Yuasa et al., (2003) in (Huyyirnah & Fitriyani, 2020) stated that sub-culture that has been carried out many times can cause the possibility of bacterial contamination, as well as decreasing or losing the pathogenicity of bacteria. The CLSI troubleshooting guide also states several things that can cause the inhibition zone to be too small or even too small, namely contamination, using magnification to read the zone, too heavy an inoculum, errors in preparing the inoculum and media thickness. Differences in the thickness of the agar media can influence the diffusion of the test substance into the agar, thereby affecting the diameter of the inhibition zone. The thicker the media used, the smaller the diameter of the inhibition zone that occurs. Agar media that complies with CLSI guidelines is 4 mm. In this research, the media should not be measured.

In Appendix 10, the results of the Kruskal Wallis test on Enterococcus faecalis bacteria, the P-value in the Asymp column. Sig. shows a value of 0.005, where the value 0.005 <0.05 which means there is a significant difference in the treatment group. This difference can be seen from the results of the Dunn test (Appendix 10) that there is a significant difference between Chlorhexidine gluconate 0.2% and all test groups. The positive control in this study, namely Chlorhexidine gluconate 0.2%, had an average inhibitory zone diameter of 5.20 ± 0.243 mm against Enterococcus faecalis bacteria. Chlorhexidine gluconate 0.2% was chosen as a positive control because it is a broad spectrum and low toxic root canal irrigation material, where its use at high concentrations (>2%) can be bactericidal which causes precipitation of bacterial cell structures, whereas its use at low concentrations (0 .2%) can be bacteriostatic, namely inhibiting the growth and development of bacteria including Enterococcus faecalis (Dini Permata Sari, Nahzi, & Budiarti, 2019) . Supported by research by Armianty & Mattulada in 2014, which proved that Chlorhexidine gluconate 0.2% has an antibacterial effect against Enterococcus faecalis bacteria.

The negative control used in this study, namely 10% DMSO, was proven to have no antibacterial activity and did not affect the test results. DMSO was chosen because it can dissolve almost all compounds, both polar and non-polar. DMSO is also a compound that has low toxicity, has anti-inflammatory and analgesic effects (Rahmi & Putri, 2020) . DMSO has the ability to penetrate cell membranes, however, when using DMSO as a solvent, the final concentration of DMSO should not exceed 10% because it can cause cell membrane rupture. The 10% DMSO solvent is an organic solvent and is not bactericidal so it cannot affect the test results (Manarisip et al., 2020) .

Research Limitations

The plants used in this research were obtained from black betel farmers in Samarinda, whose growth from planting to harvest cannot be controlled directly by researchers, and no phytochemical tests were carried out to confirm the content or secondary metabolites contained in the leaf extract.

Black betel (Piper betle L. var nigra) was used so it cannot be determined with certainty which compounds played an important role in the results of this study and how much secondary metabolites were absorbed by the solvent, and the bacteria used in this study were cultured bacteria obtained from Samarinda Regional Health Laboratory, where it cannot be known for certain how many times the bacteria have been cultured, while sub-cultures that have been carried out many times can cause the possibility of bacterial contamination, as well as decreasing or losing the pathogenicity of the bacteria.

CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that: there is antibacterial activity of black betel leaf extract (Piper betle L. var nigra) against the growth of Streptococcus mutans bacteria at concentrations of 70%, 80%, 90% and 100% with an average diameter of the inhibition zone respectively 0.50 ± 0.288 mm, 0.88 ± 0.462 mm, 1.54 ± 0.786 mm, and 1.62 ± 0.852 mm. There is also antibacterial activity of black betel leaf extract (Piper betle L. var nigra) against the growth of Porphyromonas gingivalis bacteria at concentrations of 70%, 80%, 90% and 100% with an average diameter of the inhibition zone respectively 0.44 ± 0.221 mm, 0.93 ±0.466 mm, 1.23 ± 0.633 mm, and 1.80 ± 0.901 mm. However, there was no antibacterial activity of black betel leaf extract (Piper betle L. var nigra) against the growth of Enterococcus faecalis bacteria. Further research needs to be carried out to determine the antibacterial activity of black betel leaf extract using the dilution method, as well as the antibacterial activity against bacteria other than Streptococcus mutans, Porphyromonas gingivalis, and Enterococcus faecalis. Comparative research regarding the secondary metabolite content of various types of betel leaves also needs to be carried out to understand differences in antibacterial activity test results.

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