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Antibacterial Activity of Legundi Leaf Extract (*Vitex trifolia* L.) with Betel Leaf Extract (*Piper betle* L.) against *Staphylococcus aureus*

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Abstract

One of the plants that have antibacterial compounds is legundi leaves and betel leaves. The combination of the two extracts is expected to provide a synergistic effect so that it can increase the inhibition of Staphylococcus aureus bacteria. This study used the disc diffusion test method to determine the antibacterial activity. The data obtained were analyzed using the Kolmogrov-Smirnov test and continued with the one-way Anova test. The average diameter of the inhibition zone in this study ranged from 6.43 mm to 18.63 mm where legundi extract had a medium category, betel extract had a moderate to strong category, and combined extracts had a strong category. The combination of ethanol extract of legundi leaves (Vitex trifolia Linn.) with betel leaves (Piper betle L.) has antibacterial activity against Staphylococcus aureus growth in vitro, based on the results of the one way annova test with a significance value of $p < 0.05$. The combination of antibacterial compounds from legundi with betel nut with a ratio of 2:1 had a greater inhibitory power than the two single extracts. The smallest diameter of the inhibition zone in the two leaf extracts was at a concentration of 20% (betel leaf extract had an average of 8.17 while legundi leaf had an average of 7.78). Next is a concentration of 30% (betel leaf extract has an average of 9.53 while legundi leaf has an average of 7.27) and the highest is at a concentration of 40% (betel leaf extract has an average of 14.2 while legundi leaf has an average of 8.77).

Keywords: antibacterial, legundi, betel, Staphylococcus aureus, extract

1. Introduction

Indonesia is a country that grows with tropical air and has a fairly large diversity so that it has a source of medicinal raw materials, especially traditional medicines that have been used by residents for generations with natural ingredients that are used by residents in everyday life [1][2]. According to WHO (*World Health Organization*) using plants that have effective compounds Natural ingredients sourced from past experience.

Legundi is a plant belonging to the family Verbemaceae.Legundi is a shrub or small tree that has the potential as a source of Indonesian phytopharmaceuticals [3][4]. Legundi plants contain compounds such as flavonoids, alkaloids, terpenoids, tannins, saponins, sterols (β-sitosterol and -sitosterol-β-D-glucoside), carbohydrates, proteins and amino acids [5]. Based on previous research, the compounds contained in legundi leaves are essential oils, alkaloids, terpenoids, flavonoids (persikogenin, artemetin, luteolin, penduletin, viteksicarpine, and keisosplenol-D), saponins, tannins, glucosides, proteins and carbohydrates [6]. Natural compounds that have potential as antibacterials contain flavonoids, tannins, steroids, polyphenols, terpenoids, alkaloids and saponins [7]. Legundi leaves are reported to contain phytochemicals, namely: saponins, flavonoids, and alkaloids, and have the ability to inhibit the growth of *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio para* and *Escherichia coli* bacteria in vitro [8][9].

Betel leaf plants have also been used by Indonesians for a long time as traditional medicine. Green betel leaf extract has antibacterial energy consisting of phenol and its derivative compounds that can limit various bacterial growths[10]. *Staphyloccus aureus* bacteria are normal flora in humans

as an aspect of life, but can become pathogenic if influenced by predisposing aspects [11]. The ability has advantages and advantages, one of which is that it does not have side effects so it is safer than other synthetic chemical drugs.

Staphylococcus aureus is one of the normal flora on the skin and mucous membranes, but if it is influenced by predisposing factors it can become pathogenic. *Staphylococcus aureus* is also a major cause of nosocomial infections, food poisoning and asyoktoxic syndrome, ulcers, acne, impetigo, and wound infections. Infections can be more severe including pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis and endocarditis. *Staphylococcus aureus* infection is characterized by tissue damage accompanied by abscess [12].

Research on the combination was conducted to determine whether the antibacterial activity became stronger. The mutually reinforcing effect of this combination is called the synergistic effect. This synergistic effect can be generated because the content between green betel leaf and legundi leaf has similarities, namely flavonoids, saponins and tannins. Otieno et al (2008) [13] stated that the extracts of several plants combined had greater antibacterial inhibition compared to single plant extracts. Jawetz, Melnick and Adelberg (2012) [14] also stated that when two antimicrobial agents act simultaneously on a homogeneous microbial population, the effect can be a synergistic effect. Suyasa et.al (2022) [15] mentioned a combination of betel leaf extract and legundi leaf extract in a ratio of 1: 1 has a higher inhibition zone diameter on *Staphylococcus aureus* than legundi leaf extract, but the combination of betel leaf and legundi extract is lower than betel leaf extract. Therefore, in this study, a combination of legundi and betel leaf extracts was made with a ratio of 2:1 which was then tested for its antibacterial activity against *Staphylococcus aureus* bacteria. Comparison of different extracts certainly produces different antibacterial activities so that it is known whether there is a synergistic effect given to the amount of different extracts.

2. Materials and Methods

2.1 Research Time and Place

The research was conducted in February – April 2022, at the Chemistry and Bacteriology Laboratory of the Denpasar-Bali Health Polytechnic and Agriculture Laboratory of Warmadewa University.

2.2. Research procedure

Making ethanol extract using maceration method. Betel leaves and legundi leaves are picked as much as 2 kg each. The leaves are washed, dried and crushed. The fine leaf powder was weighed as much as 200 g, then 1.5 liters of 96% ethanol were added to each. Let stand for six days while stirring using a magnetic stirrer for 8 hours every day. After six days, it was filtered and the filtrate was collected into a glass bottle, then the residue was macerated again with 500 ml of ethanol. Left for 3 days while stirring using a magnetic stirrer for 8 hours every day. After 72 hours, it was filtered and the filtrate was collected. Remaceration was carried out until the color of the filtrate was clear. The filtrate is fed into the evaporator. The extraction process is carried out automatically by evaporation at a temperature of 40-60ºC until a thick extract is obtained.

Making the concentration of betel leaf and legundi leaf extract. The extracts used, namely: single extract and a combination of extracts from legundi leaf and betel leaf with a volume ratio of legundi leaf extract with betel leaf extract of 2:1, the concentration determined in this study was 20%, 30%, 40%, with repetitions performed 3 times[16]. Dilution of betel leaf and legundi leaf extract using the following formula:

$$\% = b/vx\ 100$$

Where % is the variation of concentration (%), b is the mass of betel leaf/legundi ethanol extract (100%), and v is the volume of solvent/diluent (ethanol 96%). The amount of 100% leaf extract can be seen in Table 1. Each concentration was homogenized until the leaf extract dissolved.

Table 1.
Composition of Betel Leaf Extract and Legundi Leaf at Each Treatment Concentration

Concentration	Treatment	Material Composition		
		Betel Extract	Legundi Extract	Ethanol
20%	green betel single extract	0.3 gram		1.5 ml
	single extract legundi		0.3 gram	1.5 ml
	1:2 combination extract	0.1 gram	0.2 gram	1.5 ml
30%	green betel single extract	0.45 gram		1.5 ml
	single extract legundi		0.45 gram	1.5 ml
	1:2 combination extract	0.15 gram	0.3 gram	1.5 ml
40%	green betel single extract	0.6 gram		1.5 ml
	single extract legundi		0.6 gram	1.5 ml
	1:2 combination extract	0.2 gram	0.4 gram	1.5 ml

The media used was Mueller Hinton Agar (MHA) media which was made according to the instructions on the packaging. *Staphylococcus aureus* suspension 0.5 Mc Farlland was prepared from pure culture to which 5 ml of 0.9% physiological NaCl solution was added. The turbidity of the suspension was compared with the standard turbidity of 0.5 Mc Farlland using a densitometer.

Disc method diffusion test. Blank discs were immersed in 50 l of each extract solution which has been made. For negative control, discs soaked in 50 l 96% ethanol were used. A sterile cotton swab is dipped in the bacterial suspension. The cotton swab was scratched on the entire surface of the MHA media evenly and then allowed to stand for 15 minutes. The saturated disc is then affixed to the surface of the MHA media. Positive control used amoxicillin 30 mcg antibiotic disc. The distance between the discs is at least 15 mm. The media was then incubated at 37°C for 24 hours in an inverted position.

2.3 Data analysis

The data obtained were analyzed using the Kolmogrov-Smirnov test and continued with the one-way Anova test using SPSS version 24 software

3. Results and Discussion

The positive control used in this study was amoxicillin 30 mcg while the negative control used 96% ethanol. For 3 repetitions the diameter of the inhibition zone of the positive control was not much different, while the negative control from repetitions 1 to 3 had a value of 0 mm. The concentration of extracts observed in this study was limited to concentrations of 20%, 30% and 40% both in the single extract of betel leaf and legundi leaf and in the combination extract of the two leaves above. The results obtained can be seen in Table 2.

Table 2.
Inhibition Zone Diameter On Each Repetition

Code	Information	Replication 1	Replication 2	Replication 3	Average (mm)
a	Positive Control	37.7	34.1	36.3	36.03
b	Negative Control	0	0	0	0.00
c	Betel extract 20%	8.1	8.2	8.2	8.17
d	Legundi extract 20%	6.3	6.8	6.2	6.43
e	Combination extract 20%	17.2	16.8	17.6	17.2
f	Betel extract 30%	9.6	9.9	9.1	9.53
g	Legundi extract 30%	7.7	7.2	6.9	7.27
h	Combination extract 30%	18.3	18.5	18.7	18.5
i	Betel extract 40%	15.5	15.4	11.7	14.2
j	Legundi extract 40%	9.4	9.2	7.7	8.77

k	Combination extract 40%	18.5	18.7	18.7	18.63

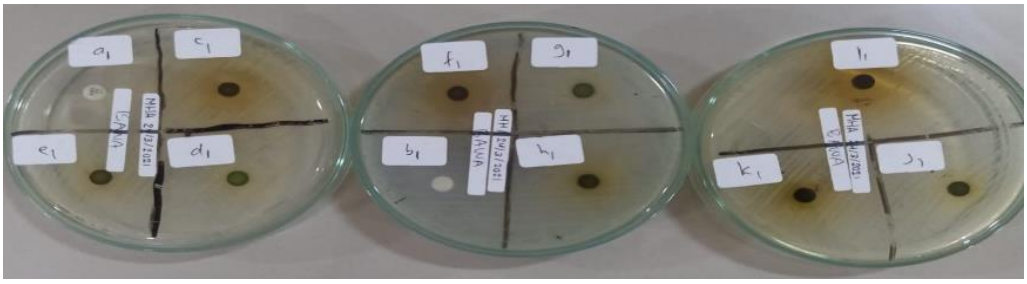

Figure 1.
One of the Results of the Inhibition Zone Test
a1. Positive Control, b1.Negative Control, c1.Combination Extract 20%, d1.Legundi Extract 20%, e1.Betel Extract 20%, f1.Combination Extract 30%, g1.Legundi Extract 30%, h1.Betel Extract 30%, i1. Combination Extract 40%, j1. Legundi Extract 40%, k1. Betel Extract 40%.

3.1 Inhibition zone category.

The average data of the diameter of the inhibition zone that was successfully studied was determined by category. Commonly used categories are inhibition zones whose values are 5 mm (weak), 6-10 mm (moderate), 11-20 mm (strong), 21 mm (very strong) [17]. The results obtained are presented in Table 3.

Judging from the categories in Table 3, the legundi extract has a medium category, the betel extract and the combination extract are classified as strong. This is due to the large concentration used in this study. Legundi extract up to a concentration of 40% is still in the medium category, if the concentration is increased, the category will change to be strong.

Table 3.
Category of *Staphylococcus aureus* Growth Inhibitory Zone

Treatment	**Average (mm)**	**Category**
Positive Control (antibiotic amoxicillin)	36.03	Very strong
Negative Control (96% ethanol)	0.00	Weak
Betel extract 20%	8.17	Currently
Legundi extract 20%	6.43	Currently
Combination extract 20%	17.2	Strong
betel extract 30%	9.53	Currently
Legundi extract 30%	7.27	Currently
Combination extract 30%	18.5	Strong
Betel extract 40%	14.2	Strong
Legundi extract 40%	8.77	Currently
Combination extract 40%	18.63	Strong

3.2 Differences in the inhibition zone of single ethanol extract with other treatments

In this study, betel leaf extract and legundi leaf extract were tested for inhibition by the disc diffusion method with concentrations of 20%, 30% and 40% respectively. The smallest diameter of the inhibition zone in the two leaf extracts was at a concentration of 20% (betel leaf extract had an average of 8.17 while legundi leaf had an average of 7.78). Next is a concentration of 30% (betel leaf extract has an average of 9.53 while legundi leaf has an average of 7.27) and the highest is at a concentration of 40% (betel leaf extract has an average of 14.2 while legundi leaf has an average of 8.77).

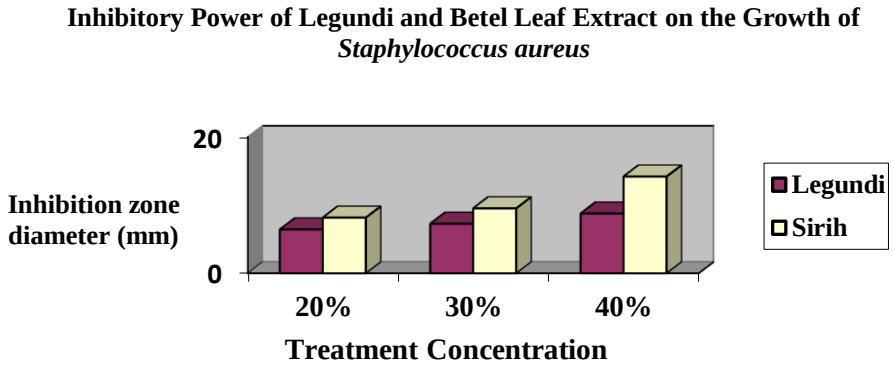

Figure 2.
Graph of Inhibitory Zone Diameter of Legundi and Betel Leaf Extract on *Staphylococcus aureus* Growth.

The difference in the zone of inhibition can be determined by the Least Significant Difference (LSD) test. In this test, most of the results obtained p value < (0.05). This indicates a significant or significant difference in the inhibition zone.

At some concentrations of extracts with other concentrations there are also those that show no difference, such as 20% betel ethanol extract compared to 40% legundi, 20% legundi ethanol extract with 30% legundi, 30% betel ethanol extract with 40% legundi, ethanol extract legundi 30% with 20% betel and 20% legundi.

3.3 The difference in the inhibition zone of the extract combination with other treatments

In Table 4, it can be seen that the smallest average diameter of the inhibition zone was at a concentration of 20% (17.20 : classified as strong), then a concentration of 30% (18.50 : classified as strong) and the highest was at a concentration of 40% (18.63 : classified as strong).

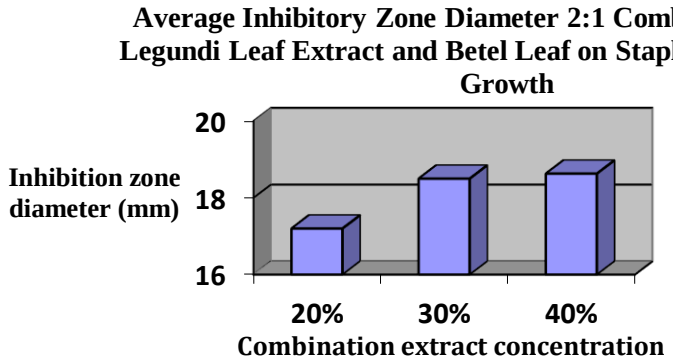

Figure 3.
Graph of Average Inhibitory Zone Diameter Combination of Legundi Leaf Extract with Betel Leaf Comparison 2:1 Against *Staphylococcus aureus* Growth

Based on the Least Significant Difference (LSD) test, it was obtained that most of the average inhibition zones of the combined extracts had a p value of <0.05, meaning that there were differences in the mean combined inhibition zones with other treatments. However, there are some average size of the inhibition zone at the combined concentration having a p value > 0.05, as in the inhibition zone the average combination of 30% with a combination of 40% and vice versa.

Thus, the hypothesis of this research is that the combination of ethanol extract of legundi leaves with betel leaf has antibacterial activity against *Staphylococcus aureus* growth in vitro. Based on the results of the one way annova test, it can be seen that the significance value is p < 0.05, this indicates that the average inhibition zone obtained in all treatments is significantly different.

In the combination study of ethanol extract of legundi leaves with betel leaf in a ratio of 2:1, the diameter of the inhibition zone every time it was repeated was always higher than the size of the inhibition zone of the constituent extracts. The average combined inhibition zone of 20% concentration was 17.2 mm, while the mean inhibition zone of betel leaf ethanol extract was 8.17 and legundi leaf was 6.43. At a combination of 30% concentration, the mean value was 18.5 mm, while the mean inhibition zone for betel nut was 9.53 and legundi was 7.27. Likewise, the combined average value of 40% was 18.63 while the mean of betel extract was 14.2 and legundi was 8.77. These results are able to answer the problems that have arisen in previous research. In a similar study using a combination extract ratio of 1:1.

The choice of a combination with a volume ratio of 2:1 between the ethanol extract of legundi with betel nut was able to increase the effectiveness and synergy of the antibacterial content. Of course, this research needs to be continued in order to find out how much this combination can be effective as an antibacterial agent that can increase the level of the inhibition zone compared to its single concentration[18].

In the study of Suyasa et al, 2022 [15], qualitatively found antibacterial compounds from ethanol extract of legundi leaves include flavonoids, tannins, phenols and quinones, while betel leaves have been investigated for the presence of flavonoid, tannin and phenol compounds[19][20]. While quantitatively, the antibacterial compounds that have just been studied are flavonoids and tannins, where the ethanol extract of legundi leaves found flavonoids 6086.53 mg/100 g QE, tannins 6752.43 mg/100g TAE. In the ethanol extract of betel leaf found flavonoid 9355.89 mg/100 g QE, tannin 42028.73 mg/100g TAE. The combination of antibacterial compounds from legundi plants with betel nut in a ratio of 2:1 has a greater inhibitory power than the single extract [21][22][23] and has been shown to have a synergistic effect [24] [25].

4. Conclusion

Based on the above research, it can be concluded that the average diameter of the inhibition zone in this study ranged from 6.43 mm to 18.63 mm where legundi extract had a moderate category, Betel extract had a moderate to strong category, and combined extracts had a strong category. The combination of ethanol extract of legundi leaves with betel leaf has antibacterial activity against *Staphylococcus aureus* growth in vitro. The combination of antibacterial compounds from legundi with betel nut in a ratio of 2:1 has a greater inhibitory power than the two single extracts. The smallest diameter of the inhibition zone in the two leaf extracts was at a concentration of 20% (betel leaf extract had an average of 8.17 while legundi leaf had an average of 7.78). Next is a concentration of 30% (betel leaf extract has an average of 9.53 while legundi leaf has an average of 7.27) and the highest is at a concentration of 40% (betel leaf extract has an average of 14.2 while legundi leaf has an average of 8.77).

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