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Comparison Of The Effectiveness Of Green Betel Leaf (Piper Betle L) And Red Betel Leaf (Piper Crocatum) Decoction Water Against Pseudomonas Aeruginosa Bacteria, One Of The Causes Of Vaginal Discharge In Vitro

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ABSTRACT

Background: Pseudomonas aeruginosa is one of the bacteria associated with vaginal discharge, and the exploration of natural antibacterial agents remains important, especially given rising antibiotic resistance. Betel leaves, both red (Piper crocatum) and green (Piper betle L), have been traditionally used for their antimicrobial properties.

Objective: To compare the effectiveness of green and red betel leaf decoction water in inhibiting the growth of Pseudomonas aeruginosa using in vitro methods.

Methods: This study employed the microdilution method to determine the Minimum Inhibitory Concentration (MIC) at various concentrations (1%, 0.5%, 0.25%, and 0.125%). Additionally, the agar diffusion method was used to assess the diameter of the inhibition zones produced by both types of betel leaf decoction against Pseudomonas aeruginosa.

Results: The red betel leaf decoction showed no inhibitory activity at any tested concentration across all replicates, indicating no detectable MIC. In contrast, the green betel leaf decoction demonstrated inhibitory effects in the first and third replicates at 1% concentration, although bacterial growth persisted in the second replicate and at lower concentrations across all replicates. Agar diffusion results revealed the presence of inhibition zones in green betel leaf decoction at 1% concentration in all replicates, while red betel leaf showed none under similar conditions.

Conclusion: Green betel leaf decoction water exhibits greater antibacterial effectiveness against Pseudomonas aeruginosa compared to red betel leaf, particularly at higher concentrations, and may serve as a potential natural alternative in managing vaginal infections.

Keywords: Green Betel Leaf (Piper Betle L), Red Betel Leaf (Piper Crocatum), Pseudomonas aeruginosa bacteria, leucorrhoea in vitro.

Keywords: *Pseudomonas aeruginosa, Green betel leaf, Red betel leaf, Antibacterial activity, Vaginal discharge*

1. INTRODUCTION

The reproductive health of women is an important aspect of global health that has a far-reaching impact on the quality of life and productivity of society. One of the common reproductive health problems is leucorrhoea, which is a major concern. According to a World Health Organisation (WHO) report, about 75% of women in the world have experienced at least one episode of vaginal discharge in their lifetime. [1] This report also notes that 65% of vaginal discharge is caused by bacterial

infection, 25% by fungal infection, and 10% by other causes[2]. The bacterium Pseudomonas aeruginosa is one of the main causes of vaginal discharge in women [3]. The research found that the bacteria causing vaginal discharge in adolescent girls were caused by Pseudomonas aeruginosa (5.88%), Klebsiella sp. (23.52%), and Hafnia alvei (5.88%) [4]. Persistent vaginal

discharge can cause discomfort and lower a woman's self-confidence. If left untreated, pathological vaginal discharge can disrupt the function of the reproductive organs, particularly the ovarian tubes, risking infertility[5].

The study found that 28.2% or 124 patients received inappropriate treatment, consisting of 2 patients in the genital skin department (1.3%) and 122 patients in the obstetrics and gynaecology department (34.0%) [6]. Metronidazole, at therapeutic concentrations, has a bactericidal effect against Pseudomonas aeruginosa. The drug is often used in the treatment of anaerobic bacterial infections, protozoan infections, and microaerophilic bacterial infections. [7]. Methronidazole, which belongs to the nitroimidazole class, is produced by Actinomycetes and Proteus. It can pass through cell membranes by passive diffusion. However, the resistance rate of metronidazole varies by geographical region, with a range between 10% and 91%[8]. Social factors also contribute to resistance, with the prevalence of infection reaching 62.7% [9]. Metronidazole works as a nitroimidazole prodrug that is activated by oxidoreductase in the cell, producing reactive compounds such as anions, nitroso, and hydroxylamine, which makes it effective in the treatment of leucorrhoea. [10].

Methronidazole works as a nitroimidazole prodrug that is activated by oxidoreductase in the cell, producing reactive compounds such as anions, nitroso, and hydroxylamine, which makes it effective in the treatment of leucorrhoea [11]. Many methods are used by the community in overcoming vaginal discharge, both pharmacological (medical treatment) and non-pharmacological, such as behavioural changes, improving personal hygiene, psychological approaches, and consumption of herbal products. Herbal medicine is considered safer because it has minimal side effects compared to modern medicine[12]. The most widely used part of the plant in traditional medicine is the leaves[13]. These bioactive compounds such as flavonoids, alkaloids, terpenoids, and phenolics have antibacterial activity against Mycobacterium species, inhibit bacterial growth and replication, and cause cell death.

There are a variety of species of betel, such as ivory betel, black betel, yellow betel, green betel, and red betel. In the treatment of leucorrhoea, green betel leaves (Piper betle L.) and red betel leaves (Piper crocatum) are often used [14]. The ingredients in betel leaves are often used to treat various health problems, such as swollen gums, vaginal discharge, mouth ulcers, dengue fever, menstrual disorders, asthma, sore throat, body odour, and nosebleeds. The leaves of red betel (Piper crocatum) have a silvery red colour with mucilage and a characteristic aroma) [18]. The leaves contain essential oils, hydroxycavicol, kavicol, kavibetol, allyprocatechol, carvacrol, eugenol, cineole, caryofelen, cadmium estragol, terpenes, and phenyl propada. The plant has antioxidant and anti-inflammatory properties[15]. The extract is also known to empirically accelerate wound healing.

Research shows that red betel leaf decoction water contains antiseptics and carvicol compounds that are disinfectants and antifungals, red betel leaf extracts and fractions have more effective tyrosinase-inhibitor activity than other fractions, although IC50 shows varying inhibitory activity The use of green betel leaf decoction is effective in reducing the incidence of leucorrhoea. [16]. Its essential oil content, including betiephenols, sesquiterpenes, starch, diastase, sugars, tannins, and alcohols, has antibacterial, antioxidant, and fungicidal properties [17]. Research comparing the antibacterial inhibition between green betel leaf extract and red betel, showed that green betel leaf extract was more effective in inhibiting the growth of Streptococcus mutans than red betel [18]. Extracts of green betel and red betel leaves extracted with ethanol have antibacterial activity against a variety of Gram-positive and negative bacteria, including Pseudomonas aeruginosa[19]. Extracts of green and red betel were able to inhibit the growth of Pseudomonas aeruginosa at concentrations of 10% and 15%[20].

According to the description that has been presented, this study aims to compare the effectiveness of green betel leaf (Piper betle L.) and red betel leaf (Piper crocatum) decoction water against Pseudomonas aeruginosa, the cause of vaginal discharge, in vitro. This research uses fresh plant material with water as the main solvent so that the results can be applied by the community.

2. METHOD

This was an experimental laboratory study conducted with several treatments for the experimental group and a control group for comparison. The method used is in vitro with dilution technique, which aims to determine the potential of a compound in inhibiting bacterial growth through the determination of Minimum Inhibitory Concentration (KHM). In this case, the microdilution method was used to determine the KHM value and compare the effectiveness of green betel leaf (Piper betle L) and red betel leaf (Piper crocatum) boiled water against the growth of Pseudomonas aeruginosa bacteria. This research was conducted at the Microbiology Laboratory of the Faculty of Pharmacy, Hasanuddin University on 11-29 November 2024. Tools used included analytical balance, timer, micropipette, Erlenmeyer, vortex, ose, test tube, microplate, petridisk, incubator, 10 cc spoit, spiritus, autoclave, oven, caliper, Mc.Farland densitometer, and various glassware. Materials used included green and red betel leaf cooking water, Pseudomonas aeruginosa bacteria, amoxicillin, sterile distilled water, MHA (Muller Hinton Agar), 0.1% TCC, and 10% DMSO solution.

The process began with the sterilisation of tools and materials using an autoclave at 121°C for 1 hour, while the petri dishes were sterilised in an oven at 170°C. The green and red betel leaves that had been picked, washed, drained, and cut into small pieces were then boiled at 100°C for 10 minutes in a ratio of 1:4 water. The decoction was filtered using a filter cloth, stored

in a tightly closed container, and dried using the freeze drying method. The resulting lyophilisate powder was weighed and made a test stock solution with a concentration of 10% (0.5 grams of lyophilisate in 5 mL of 10% DMSO). The bacterial rejuvenation was carried out by inoculating Pseudomonas aeruginosa onto the inclined agar medium and incubated at 37°C for 24 hours. After that, bacterial suspension was made by dispersing the bacteria in NaCl until it reached McFarland turbidity standard 0.5 or equivalent to 10^8 CFU/mL. The stage of KHM testing was carried out using the microdilution method with a 48-well microplate. Each well was filled with 500 µL of mixture consisting of 445 µL media, 50 µL test solution, and 5 µL bacterial suspension. Multilevel dilutions (1%, 0.5%, 0.25%, 0.125%) were performed, then incubated at 37°C for 24 hours. After incubation, each test solution was added 0.1% TCC and allowed to stand for 15 minutes to observe the colour change. If the solution remains clear, it means that bacterial growth is inhibited, while if it is red, it means that bacterial growth is not inhibited. If the result is not clear, a confirmation test is carried out by scratching the solution on solid media and incubated again. Furthermore, the diffusion method was used to compare the effectiveness of green and red betel leaf cooking water against Pseudomonas aeruginosa. The bacterial suspension was diluted to 10^7 CFU/mL, then spread on NA media using a cotton swab. Paper discs with green betel leaf, red betel leaf, positive and negative control test solutions were placed on the media and incubated at 37°C for 24 hours. The zone of inhibition was measured using a caliper, where the larger the clear zone, the more effective the inhibition of the test solution. The data were analysed descriptively by collecting and interpreting the experimental results directly [21]. This method was chosen because it can show the effectiveness of green and red betel leaf cooking water in inhibiting the growth of Pseudomonas aeruginosa, which causes vaginal discharge.

3. RESULTS AND DISCUSSIONS

RESULTS

1. A Decoction of Red Betel (Piper crocatum) and Green Betel (Piper betle L) Leaves

Extraction method used in this study was boiling for 10 minutes, followed by lyophilisation process to obtain dry extract. This method refers to previous research which states that heating for 10 minutes provides optimal results on the effectiveness of the compound content in the tested solution [22]. In this study, 227 grams of red betel leaves were used to produce 1.13 litres of decoction. After the freeze dryer process, 4.43 grams of dry extract or lyophilisate was obtained. Meanwhile, 250 grams of fresh green betel leaves produced 1.25 litres of decoction, which after going through the freeze dryer process produced 9.70 grams of lyophilisate. The percentage yield obtained shows that red betel leaves have a yield of 1.95%, while green betel leaves have a yield of 3.88%. This shows that green betel leaves produce more dry extract than red betel leaves, as shown in the table below.i;

Table 3.1 Yields of Red Betel Leaf and Green Betel Leaf

Decoction	Sample (gram)	Lyophyllicate (gram)	Yield (%)
Red betel leaf	227	4,43	1,95 %
Green betel leaf	250	9,70	3,88 %

Source: Bethel leaf yield

From table 3.1 it can be seen that the yield of red betel leaf yield is 1.95% and the yield of green betel leaf yield is 3.88%.

2. Bethel leaf soak results

The Minimum Inhibitory Concentration (KHM) is the lowest concentration that causes a decrease in absorbance value after incubation, which indicates the inhibition of bacterial growth [23]. The results of the antibacterial test showed that red betel leaf did not have a KHM because there was still bacterial growth in all replicates and concentrations. Meanwhile, in green betel leaf, the antibacterial test showed that at a concentration of 1% in the first and third replications no bacterial growth was found, although in the second replication there was still bacterial growth. At concentrations of 0.50% to 0.125%, bacteria still grew in all replicates. Thus, it can be concluded that the KHM for green betel leaf against Pseudomonas aeruginosa bacteria is 1%. After knowing the KHM, the number of green betel leaves needed as antibacterial was calculated. Based on the calculation, the weight of leaves needed to inhibit bacteria is 25.77 grams. If one green betel leaf weighs about 2.1 grams, then the number of leaves needed is about 12 sheets.

Table 3.2 Minimum inhibitory concentration of red betel leaf and green betel leaf against Pseudomonas aeruginosa bacteria

Replication	Red Bethel Leaves				Green Bethel Leaves			
	1 %	0.50%	0,25%	0,125%	1%	0.50%	0,25%	0,125%
I	+	+	+	+	-	+	+	+
II	+	+	+	+	+	+	+	+
III	+	+	+	+	-	+	+	+

Sources: Observation of minimum inhibitory concentration

Remarks: Sign (+) there is bacterial growth and sign (-) there is no bacterial growth Pseudomonas aeruginosa.

Figure 3.2 shows that the results of the red betel leaf antibacterial test in all replicates at all concentrations, there is still bacterial growth so there is no minimum inhibitory concentration (KHM). While the results of the green betel leaf antibacterial test in replicates I and III at a concentration of 1% there was no bacterial growth. However, at a concentration of 1% in the second replication and concentrations of 0.50% to 0.125% both in replications I, II and III there is still bacterial growth. So it can be concluded that the minimum inhibitory concentration of green betel leaves on Pseudomonas aeruginosa bacteria is 1%.

1. Inhibition Test of Red Betel Leaf and Green Betel Leaf

The inhibitory power test was carried out by agar diffusion method to observe the inhibition zone of Pseudomonas aeruginosa bacterial growth. The results showed that at a concentration of 10%, green betel leaf had an average inhibition zone diameter of 10.83 mm with a standard deviation of 1.34 mm. Meanwhile, red betel leaf only had an average inhibition zone of 3.28 mm with a standard deviation of 4.65 mm. This indicates that green betel leaves are more effective in inhibiting bacterial growth compared to red betel leaves.

There was no zone of inhibition in the positive control using Amoxicillin and the negative control using 10% DMSO. This indicates that the observed antibacterial effect is from the active ingredients in the green betel leaf extract and not from other factors.

Figure 3.3 Diameter of inhibition zones of red betel leaf and green betel leaf lyophilisates

Test Sample	Concentration inhibition zone diameter 10%			Average ± DS
	Treatment I	Treatment II	Treatment III	
Red betel leaf	0	9,89	0	
	0	10,3	0	3.28± 4.65
	0	9,39	0	
Green betel leaf	9,85	10,66	12,17	
	10,14	11,66	11,45	10.83 ± 1.34
	9,25	10,91	13,67	
Positive control with *Amoxicillin*	0	0	0	
	0	0	0	0 ± 0
	0	0	0	
negative control with DMSO 10%	0	0	0	
	0	0	0	0 ± 0
	0	0	0	

Sources: Frequency Distribution Table (Abdul Wahab, 2021) the results of measuring the diameter of the inhibition zone using the agar diffusion method

This table shows the difference in inhibition between red betel leaf lyophilisate and green betel leaf at a maximum concentration of 1%. In green betel leaves, the average inhibition zone is 10.83 mm with a standard deviation of 1.34 while in red betel leaves the average inhibition zone is 3.28 and a standard deviation of 4.65 which means that green betel leaves are more effective in inhibiting bacterial growth. Then in the positive and negative controls no inhibition zone was found.

4. DISCUSSION

1. Yield of Red Betel Leaf and Green Betel Leaf

Based on the extraction results, the yield of red betel leaves was 1.95%, while the yield of green betel leaves reached 3.88%. In addition, the yield of lyophilisate obtained from red betel leaves was 4.43 grams, while green betel leaves were 9.70 grams. The smaller yield in this study was due to the use of young betel leaves. Other factors that affect the difference in yield include the content of active substances, the age of the plant, and the location of plant growth. Yield of Red Betel Leaf and Green Betel Leaf

Furthermore, differences in extraction yield can be influenced by various variables such as extraction time, extraction method, part of the sample used, type of solvent, sample to solvent ratio, extraction temperature, and sample particle size. The age of betel leaves also plays an important role in determining the yield and essential oil content. Fresh betel leaves (young) have a higher essential oil content compared to older betel leaves [24].

2. Minimum Inhibitory Concentration (KHM) of Red Betel Leaf and Green Betel Leaf Lyophilisates

The results showed that the lyophilisate of green betel leaf decoction water at a concentration of 1% was able to completely inhibit the growth of Pseudomonas aeruginosa, as evidenced by the absence of bacterial growth in all three repetitions. This indicates that green betel leaf extract has strong antibacterial activity in vitro. The content of active compounds such as flavonoids, tannins, and essential oils play a role in antibacterial mechanisms, for example by damaging bacterial cell membranes, inhibiting protein synthesis, or disrupting bacterial metabolism. Being a Gram-negative bacterium, Pseudomonas aeruginosa has a complex outer membrane structure, which often makes it resistant to various antibacterial agents. However, the results of this study suggest that the active compounds in green betel leaf are effective enough to overcome these challenges. These findings provide a scientific basis for the development of green betel leaf as an alternative herbal therapy in the treatment of Pseudomonas aeruginosa infections. In addition, further studies are needed to ensure its safety, stability and effectiveness under in vivo conditions, as well as exploring the molecular mechanisms underlying its antibacterial activity.

This research result is in line with a study that found that green betel leaf extract (Piper betle L.) has antibacterial activity as indicated by the formation of a clear zone in the agar diffusion test. At concentrations of 10% and 15%, the inhibition zones produced were 11.2 mm and 14.5 mm, respectively. Meanwhile, research on the antibacterial activity of red betel leaf (Piper crocatum) lyophilisate against Pseudomonas aeruginosa showed that red betel leaf extract contains active compounds such as flavonoids, tannins, saponins, and alkaloids, which are known to have antibacterial activity. However, in this study, lyophilisate of red betel leaf cooking water was not effective enough in inhibiting the growth of Pseudomonas aeruginosa at the concentrations tested. The extract of red betel leaf has antibacterial activity against Pseudomonas aeruginosa, with a Minimum Inhibitory Level (KHM) at a concentration of 10% [25], and also has antimicrobial activity against Pseudomonas aeruginosa, with the highest inhibition at a concentration of 50% [26].

3. Inhibition Test of Red Betel Leaf and Green Betel Leaf

Betel leaf green has stronger antibacterial potential than betel leaf red, especially at a concentration of 1%. This can be seen from the formation of growth inhibition zones in the first, second and third repetition tests for green betel leaves, while red betel leaves with the same concentration still showed bacterial growth. Thus, the lyophilisate of green betel leaf decoction water at 1% concentration was able to inhibit or even kill Pseudomonas aeruginosa significantly, making it a potential candidate for the development of herbal-based antimicrobial therapy.

The comparison between green betel leaf and red betel leaf in inhibiting the growth of Pseudomonas aeruginosa showed that green betel leaf was superior, especially at 1% concentration. However, this result is different from a study that concluded that red betel leaf extract can inhibit the growth of Pseudomonas aeruginosa in vitro [27]. Essential oils found in green betel leaves have higher antibacterial activity compared to red betel leaves, although both have similar chemical components [28].

The research exploring the antibacterial activity of green betel leaf (Piper betle L.) and red betel leaf (Piper crocatum) against various pathogenic bacteria showed that green betel leaf extract at concentrations of 25%, 20%, and 15% was effective in inhibiting the growth of Pseudomonas aeruginosa, with a significant decrease in Optical Density (OD) values, while concentrations of 10% and 5% did not show the same effectiveness [29]. Overall, this study confirmed that green betel leaves have stronger antibacterial ability against Pseudomonas aeruginosa compared to red betel leaves. Therefore, green betel leaf is more promising to be developed as an alternative herbal-based therapy to treat infections caused by this bacteria.

Standardisation of Green Betel Leaf Extract (Piper Betle L.) and Antibacterial Test Against Pseudomonas aeruginosa

Bacteria concluded that green betel leaf extract meets drug standards and has the ability to inhibit the growth of Pseudomonas aeruginosa bacteria [30]. In addition, research shows that green betel leaf extract (Piper betle L.) can inhibit bacteria at concentrations of 15%, 20%, and 25% [31]. This is also in line with research that found that ethanol extracts of green betel leaves have active antibacterial properties [32].

Differences in the effectiveness of green betel leaves and red betel leaves in this study were also caused by differences in essential oil content. According to research by Lidwina Ella (2024), the essential oil content in dark green betel leaves is higher (0.20%) compared to dark red betel leaves (0.16%). The higher the concentration of essential oil, the stronger the antibacterial power against Pseudomonas aeruginosa. The study found that red betel leaf extract had moderate inhibition against Pseudomonas aeruginosa at 50% concentration, with an average inhibition zone of 8.7 mm [33].

5. CONCLUSION

Based on the results of the study, it can be concluded that green betel leaf (Piper betle L.) lyophilisate has a minimum inhibitory concentration (KHM) of 1%, while red betel leaf (Piper crocatum) lyophilisate does not show KHM against Pseudomonas aeruginosa. This shows that green betel leaf decoction water is more effective than red betel leaf in inhibiting the growth of Pseudomonas aeruginosa, a bacterium that plays a role in causing vaginal discharge. Future research is expected to test the KHM and inhibitory power of lyophilisate of red betel leaf decoction water with concentrations above 1% using the same in vitro method. In addition, in vivo studies are also needed to support the development of betel leaf-based products as an alternative treatment for leucorrhoea. For the public, it is recommended to boil 12 pieces of green betel leaves with 100 ml of water for 10 minutes or 120 pieces with 1 litre of water, which can then be used to wash the external genital area as a treatment for leucorrhoea (fluor albus).

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