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Antibacterial potential ethanol extract of beluntas leaves (*Pluchea indica L*) to *Streptococcus sanguinis*

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ABSTRACT. Recurrent aphthous stomatitis (RAS) is one of the most common oral diseases in the community, with a prevalence of 5-66%, with one of the predisposing factors being *Streptococcus sanguinis*. Treatment for RAS has been symptomatic and supportive, including antiseptic mouthwash such as chlorhexidine gluconate 0.2% or topical corticosteroids (triamcinolone acetonide 0.1% in Orabase). However, these drugs have some side effects. Treating herbal ingredients such as Beluntas leaves low prices and minimal side effects. The active compounds in Beluntas leaves are phenols, tannins, flavonoids, saponins, triterpenoids, essential oils, terpenoids, and many compounds known to have antibacterial activity. Methods: This study aimed to determine the minimum inhibitory level (MIC), and minimum killing rate (MBC) of 96% ethanol extract of Beluntas leaves on the growth of *Streptococcus sanguinis*. MIC was measured by broth microdilution technique with DMSO solvent 10% and eight concentrations of beluntas extract. Chlorhexidine gluconate 0.2% was used as a positive control for the comparison compound. Furthermore, the MBC test was carried out using the total plate count method for treatments that gave the MIC value. One Way Anova analysis with Post Hoc Tukey was used to determine the significant difference between treatments. Results: The ethanol extract of Beluntas leaves (*Pluchea indica L*) has a Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for the growth of *Streptococcus sanguinis* with a minimum inhibitory concentration of 3.95 g/mL and a minimum concentration of 7.8 g/mL. Conclusion: The ethanol extract of Beluntas leaves (*Pluchea indica L*) has the potential as an antibacterial against *Streptococcus sanguinis*.

KEYWORDS: Recurrent aphthous stomatitis, *Streptococcus sanguinis*, ethanol extract of beluntas leaves, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration

INTRODUCTION

Recurrent aphthous stomatitis (RAS) is a problem that often arises in dentistry and is one of the most common oral diseases in the community, with a prevalence of 66%. RAS is characterized by recurrent ulcers on the patient's oral mucosa and can be painful. The prevalence of oral ulceration worldwide is as much as 4%, where RAS is the disease with the greatest prevalence of 25% and is experienced by most women in the second and third decades.[1,2]

The clinical manifestations of RAS are ulcers, single or multiple, shallow, oval, and pain.[3] Prodromal symptoms appear before the onset of RAS, including discomfort and redness for 1-3 days.[4] The exact etiology of RAS has not been determined so the research focused on the predisposing factors for RAS. Several factors are considered predisposing factors for RAS, including immunological abnormalities, genetics, systemic factors, endocrine system, stress, smoking cessation, allergic factors, and microorganisms. The main microorganisms associated with the formation of RAS are pleomorphic transitional L α-hemolytic Streptococcus and *Streptococcus sanguinis*.[5]

One of the efforts to manage RAS is by symptomatic and supportive treatment because, until now, the etiology of RAS is still unknown. Therefore, treatment is only aimed at curing complaints. The goal of symptomatic treatment is to reduce symptoms and the number and size of ulcers. Currently used drugs include: antiseptic mouthwash (chlorhexidine gluconate 0.2%) to reduce the

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duration and discomfort of RAS or topical corticosteroids (triamcinolone acetonide 0.1% in Orabase).[6] Chlorhexidine gluconate 0.2% is the gold standard, effective against gram-negative and positive and facultative aerobes and anaerobes. However, its use in more than two weeks has side effects, including causing burning of the oral mucosa, impaired taste, tooth staining, erosion of the oral surface, and dryness of the oral cavity.[7]

Therefore, it is necessary to develop an alternative therapy by utilizing herbal plants in curing ulcers in RAS. It is intended so that patients can recover without side effects at a relatively low cost.[8] The development of a back-to-nature lifestyle has made herbal plants increasingly widespread by the community as alternative materials for treating or preventing certain diseases. The community considers that herbal ingredients are easier to obtain, the price is more affordable, and the side effects are minimal, so it is safer for consumption.[9] One of the natural ingredients that can be used in traditional medicine is the beluntas plant.[10] The leaves, flowers, and roots of beluntas can be used for therapy, but the beluntas leaves are the part that has the highest biologically active component.[11]

Beluntas (*Pluchea indica L*) is a wild plant in dry areas on hard and rocky soils or grown as a hedge.[12] This plant has a distinctive aromatic odor and bitter taste. The part used from this plant is the leaves and roots, which are efficacious for eliminating body odor and bad breath, increasing appetite, overcoming digestive disorders in children, relieving pain in rheumatism, and so on. The active compounds in beluntas leaves are flavonoids, triterpenoids, phenols, and essential oil derivatives.[13] Beluntas leaves have antibacterial properties due to the content of phenolics, triterpenoids, and tannins that can cause bacterial cell death.[14,15]

Based on several studies that have been carried out, the ethanol extract of beluntas leaves (*Pluchea indica L*) has been shown to inhibit bacterial growth. These bacteria include Streptococcus mutans, Staphylococcus aureus, Bacillus subtilis, and *Pseudomonas aeruginosa*.

Based on the explanation above and there is no research on *Streptococcus sanguinis* bacteria, so the authors are interested in research to determine the antibacterial effect of beluntas leaves through testing the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the ethanol extract of the beluntas leaves on the growth of *Streptococcus sanguinis* which is one of the bacteria that causes recurrent aphthous stomatitis.

MATERIALS AND METHODS

Determination and Ethanol Extract of Beluntas Leaves

Beluntas leaves were obtained from the Subang Tropical Balitbu plantation, which was then determined at the Biology Laboratory of Padjadjaran University. Extract beluntas leaves with 96% ethanol and let stand for three days, then the extract is filtered using filter paper to obtain the filtrate. Then the filtrate was evaporated using a rotary evaporator at a temperature of 70°C until the consistency was like a paste.

The ethanol extract of beluntas leaves was put into a 15 mL tube with as much as 0.5 grams and dissolved in 10% DMSO to reach a concentration of 500 g/mL. The extract was diluted using a multichannel Micropipette so that the final concentrations were 250g/ml, 125g/ml, 62.5g/ml, 31.3g/ml, 15.6g/ml, 7.8g/ml, 3.95g/ml, and 1.95g/ml.

Preparation of *Streptococcus sanguinis* inoculum

The bacteria used in this study is *Streptococcus sanguinis* ATCC 10556. Take two oses and suspension in BHI-B, incubated for 24 hours. Then standardized with 0.5 McFarland solution, the total density of bacteria used is 1.5 x 10^8 CFU/ml. Check using a spectrophotometer with a wavelength of 600nm, then dilute to 10^5.

Minimum Inhibitory Level (MIC) and Minimum Killing Rate (KBM)

The method used in this study followed the method developed by CLSI (Clinical Laboratory Standard Institute) with a slight modification, namely adding 2% sucrose to BHI-A and BH-B media.

This study consisted of ten treatments, namely eight treatments of ethanol extract of beluntas leaves with concentrations of 250g/ml; 125g/ml; 62.5g/ml; 31.3g/ml; 15.6g/ml; 7,8g/ml; 3.95g/ml, and

1.95g/ml; positive control (chlorhexidine gluconate 0.2%); and negative control (DMSO).

200 L/mL of BHI-Broth media was added to all 96 well plates and added 200 l of ethanol extract of beluntas leaves of each concentration, and 10 l of bacterial suspension, with the format of media+sample (negative control), media+solvent (solvent control), media+sample+bacteria (test sample), media+solvent+bacteria (positive control). Incubate at 37°C for 16-20 hours. Insert the well plate into a spectrophotometer with a wavelength of 600 nm to see the minimum inhibitory concentration using the absorbance method.

Take 200 L of 0.2% Chlorhexidine gluconate and put it into the well plate with 3 repetitions, 10 l of bacterial suspension 10^5 for two repetitions, and one repetition plate without bacteria as a positive control. Incubate for 24 hours at 37°C with 5% CO_2.

96-well plates indicated as MIC and KBM were inoculated into sterile BHI-Agar medium in Petri dishes and incubated at 37°C with 5% CO_2 for 24 hours, calculating the number of colonies in each petri dish from each treatment with various concentrations of extract using a colony counter.

The number of colonies contained in BHI-Agar media was used to determine MIC and MBC was calculated using a Colony counter. The calculation of the number of colonies formed is called the total plate count (TPC), with colony forming units/mL (CFU/mL) calculated by the formula:

$$\text{TPC value (CFU/ml)} = \frac{\text{Number of colonies formed x Dilution factor}}{\text{The volume of bacterial colonies that is included in the criteria}}$$

The formula measures the calculation of the percentage of bactericidal power:

$$\%\ \text{Kill Efficiency} = \frac{\text{TPC bacteria control (CFU/ml)} - \text{TPC treatment (CFU/ml)}\ x100\%}{\text{Mean bacterial colony control}}$$

RESULTS

The Beluntas plants' determination results at the Biology Laboratory of Padjadjaran University, Jatinangor, and West Java showed that the sample used was Beluntas (*Pluchea indica L*). Phytochemical tests were carried out at the Central Laboratory of Padjadjaran University, Jatinangor, West Java. Table 1 shows the results of qualitative phytochemical tests taken from the ethanol extract of beluntas leaves.

Table 1. Qualitative Phytochemical Test Results

No	Secondary Metabolite	Method	Results
1	Phenolic	Reagent $FeCL_3$ 5%	++
2	Tanin	Reagent $FeCL_3$ 1%	++
3	Flavonoid	a. Reagent HCL concentrated + Mg	-
		b. Reagent H_2SO_4 2N	-
		c. Reagent NaOH 10	+
4	Saponin	Heated	+
5	Triterpenoid	Reagent H_2SO_4 concentrated + CH_3COOH	++
	dan Steroid	anhydrous	-
6	Alkaloid	Reagent Dragendorff	+

Description: +: Little; ++: Medium; +++: Much ; - : None

Minimum Inhibitory Level (MIC)

The results of the MIC test observed the growth of *Streptococcus sanguinis,* which was indicated by turbidity at the bottom of the well in several treatments compared to negative controls. The results of visual observations can be seen in the good extract of beluntas at a concentration of 1.95g/mL. There is turbidity at the bottom which is a culture of *Streptococcus sanguinis*. There was slight

turbidity at well 3.9g/mL, while at wells 7.8 to 250, visually, no difference in turbidity was observed. Solvent control and clear positive control showed no contamination in the experiment.

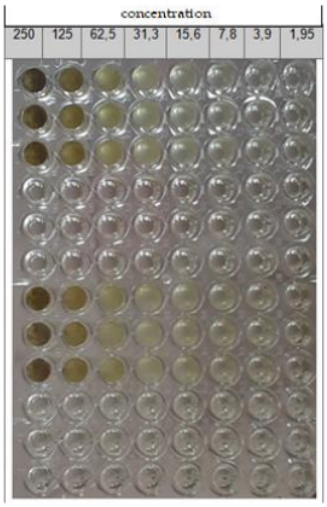

Figure 1. The plate of minimum inhibitory content test results

The average results of the absorbance of the effect of the ethanolic extract of beluntas leaves on the number of colonies of *Streptococcus sanguinis* using spectrophotometry with a wavelength of 600nm, resulting in quantitative data that confirmed that the MIC was right at a concentration of 3.9 g/mL. (**Table 2**)

Table 2. The absorbance of ethanol extract of beluntas leaves against *Streptococcus sanguinis*

Extract Concentration	Media control	Average Absorbance Bacteria+ Sample	Difference	Note(s)
250µg/mL	2,039	2,018	0,021	
125µg/mL	1,4203	1,3964	0,023	
62.5µg/mL	0,8977	0,8568	0,04	
31.25µg/mL	0,5208	0,4938	0,27	
15.62µg/ mL	0,312	0,2874	0,25	
7.8µg/mL	0,1873	0,1789	0,84	
3.9µg/mL	0,1245	0,114	0,1	MIC
1.95µg/mL	0,0795	0,0833	-0,0035	

The results of the positive control test (chlorhexidine gluconate 0.2%) obtained the average number of colonies of *Streptococcus sanguinis* bacteria with three repetitions after 24 hours of incubation was 0 CFU/mL with an average bactericidal power of 100%.

Minimum Bactericidal Concentration (MBC)

The results of the MBC test showed that at a concentration of 7.8g/mL, the ethanol extract of the tested beluntas leaves was not observed for the growth of *Streptococcus sanguinis*. To count the number of bacteria at each test concentration, the number of bacteria was calculated using the total plate count technique with a colony counter, as shown in **Figure 2** and **Table 3**.

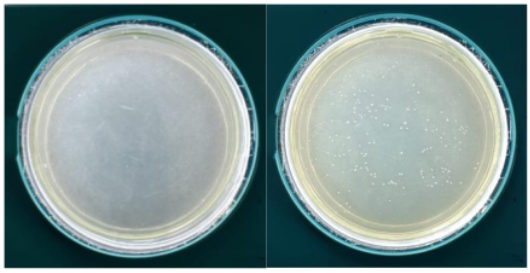

Figure 2. The results of the calculation of the number of bacteria using the Colony Counter
Notes: (A) Number of *Streptococcus sanguinis* Bacteria Colonies at the Concentration of Ethanol Extract of Beluntas Leaves 7.8g/mL (KBM). (B) Number of *Streptococcus sanguinis* Bacteria Colonies at Concentration of Ethanol Extract of Beluntas Leaves 3.9g/mL (MIC)

Table 3. Number of *Streptococcus sanguinis* Bacteria Colonies After 24 hours of incubation at various concentrations

Extract Concentration	Mean Colony (CFU/mL)	Mean kill Efficiency	Note(s)
Group A (250 µg/mL)	0	100%	
Group B (125 µg/mL)	0	100%	
Group C (62.5 µg/mL)	0	100%	
Group D (31.25 µg/mL)	0	100%	
Group E (15.62 µg/ mL)	0	100%	
Group F (7.8 µg/mL)	0	100%	MBC
Group G (3.9 µg/mL)	$12{,}9 \times 10^{1}$	99% (0,9999612)	MIC
Group H (1.95 µg/mL)	$27{,}3 \times 10^{5}$	99% (0,9999178)	

Colony Number Normality Test Results

The normality test results using Shapiro-Wilk showed that two variables were tested, namely concentrations of 3.95g/mL and 1.95g/mL. It is known that the significance value is 0.147 and 0.114, where the value is more significant than with a value of 0.05. Thus, it can be concluded that the data is normally distributed.

One-Way ANOVA Analyses

The results of the One Way ANOVA test to determine whether the data has an average difference or cannot be known from the significance value of the number of colonies then compared with the significance value, with a test decision, if the value (Sig) > 0.05, then there is no difference in average if value (Sig) < 0.05 then there is an average difference. The results of the ANOVA test above show the F value for the number of colonies of 22.882 and the significance (p-value) of 0.000 is less than 0.05, so it can be concluded that at concentrations of 3.95 and 1.95, there was a different effect on the number of colonies.

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Tukey's Post-Hoc Test Results

Based on the results of the Tukey Post Hoc test in appendix 3, the number of colonies at a concentration of 250 was the same as a concentration of 125, 62.5, 31.3, 15.6, 7.8, and 1.95 because they were in the same subset. Meanwhile, the number of colonies at a concentration of 3.95 was not the same as that of 125, 62.5, 31.3, 15.6, 7.8, and 1.95 because they were indifferent subsets. The comparison results can be seen through the mean difference column, obtained at a concentration of 3.95 significantly different ($p < 0.05$).

DISCUSSION

Based on the research results presented in **Table 2**, it was found that the ethanol extract of beluntas leaves had an inhibitory and killing effect on the growth of *Streptococcus sanguinis* bacteria and there was MIC at a concentration of 3.9g/mL with 99% killing power and MBC was found at a concentration of 7.8g/mL. mL with 100% killing power. The positive control group of chlorhexidine gluconate at a concentration of 0.2% had an inhibitory and killing effect on *Streptococcus sanguinis* bacteria with an average killing power of 100%. Therefore, it can be said that the ethanol extract of beluntas leaves at a concentration of 7.8g/mL had the same antibacterial effect as 0.2% chlorhexidine gluconate. The statistical test value obtained a P-value of 0.00, which states that the MIC and MBC values significantly affect the growth of *Streptococcus sanguinis* bacteria.

These results follow previous research, which stated that the ethanol extract of beluntas leaves (*Pluchea indica L*) could inhibit the growth of bacteria, *Staphylococcus aureus*, *Bacillus subtilis,* and *Pseudomonas aeruginosa*.[16,17] Based on the results of qualitative phytochemical tests, active compounds in beluntas leaves, such as phenols, tannins, flavonoids, saponins, triterpenoids, and alkaloids. Phenol compounds have antibacterial properties due to the presence of hydroxyl and carbonyl groups that can interact with bacterial cells through hydrogen bonds. It will cause the coagulation of proteins and bacterial cell membranes, which will cause bacteria to lyse.[18] Phenol can also cause hyperpolarization of the cytoplasmic membrane and increase the instability membrane. It causes membrane dysfunction and bacterial cell death.[14,15]

Tannins are one type of compound that belongs to the polyphenol group and has soluble properties in water and organic solvents. Tannins can be used as antibacterial because they have an antiseptic phenol group, so they can be used as an antibacterial component through hydrogen bonds which will form complex compounds with proteins and will cause bacterial cell proteins to be denatured so that bacterial metabolism is disrupted. [14,15,19]

Flavonoids also have a role as antibacterial compounds by inhibiting cell membrane function, nucleic acid synthesis, and inhibiting energy metabolism. The position in inhibiting the synthesis of nucleic acids occurs through the accumulation of nucleic acid bases, thereby inhibiting the formation of DNA and RNA because rings A and B of flavonoid compounds play an important role in the process of intercalation or hydrogen bonding. The result of flavonoid interaction will also cause damage to the permeability of the bacterial cell wall. Flavonoids will also form complex extracellular compounds and soluble proteins that cause damage to cell membranes and the release of intracellular compounds, thus causing cell function to be inhibited. In addition, energy metabolism will also be hampered due to the inhibition of oxygen used by bacteria. It can occur by preventing the formation of energy in the cytoplasmic membrane and inhibiting the motility of bacteria which play a role in antimicrobial activity and extracellular proteins.[20,21]

Other components, namely saponins, have broad biological activity and act as antibacterial and antifungal agents. Saponins can cause leakage of bacterial cell membranes, resulting in damage to membrane permeability which can interfere with the survival of bacteria. When the stability of the bacterial cell membrane is disturbed, it will cause the cytoplasm to come out of the cell. It occurs when saponins bind to the cytoplasmic membrane through the outer membrane, which will cause the stability of the bacterial cell membrane to be disturbed and result in bacterial cell lysis and then death. [21,22]

Triterpenoid compounds are also reported to have antibacterial activity. Triterpenoids will react with porins (transmembrane proteins) on the outer wall of the bacterial cell membrane causing the formation of strong polymer bonds. This condition will cause damage to the porin, which is the entrance and exit of the compound. Furthermore, this causes the permeability of the bacterial cell wall to decrease, the bacterial cell to lysis, and the death of the bacterial cell. [14,15,23,24]

Alkaloids also have antibacterial properties that cause bacterial cell death because the cell wall layer is not completely formed by interfering with the peptidoglycan constituent components of bacterial cells by interfering with the formation of cross-bridges of peptidoglycan constituent components in bacterial cells. Alkaloids can also inhibit enzymes that play a role in the DNA replication process, inhibiting bacterial growth because bacteria cannot divide by inhibiting DNA replication.[25,26]

Because beluntas leaves have an active biological content that acts as an antibacterial, the ethanol extract of beluntas leaves can be developed and used as natural antibacterial ingredient because there is a significant value at a concentration of 7.8g/mL with the number of colonies of *Streptococcus sanguinis* bacteria 0 CFU/mL.

CONCLUSION

The ethanol extract of beluntas leaves (*Pluchea indica L*) has the potential as an antibacterial against *Streptococcus sanguinis*.

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