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Demikian agar tugas ini dilaksanakan dengan sebaik-baiknya.

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Minimum inhibitory concentration and minimum bactericidal concentration of beluntas leaf ethanol extract against Streptococcus mutans

Elin Septiani,[1] Natallia Pranata,[2] Vinna K Sugiaman[2*]

Abstract

Objective: The purpose of this study was to determine the exact values of Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) of beluntas leaf extract against Streptococcus mutans colonies.

Material and Methods: This study used Streptococcus mutans ATCC 25175 and ethanol extract of beluntas leaves with concentrations: 250 g/ml, 125 g/ml, 62.5 g/ml, 31.3 g/ml, 15.6 g/ml, 7.8 g /ml, 3.9 g/ml and 1.95µg/ml. This study used two methods, broth microdilution and total plate count techniques as the designated method for the purpose of counting bacteria. Furthermore, the data analysis test was performed with one way ANOVA parametric test to determine the significance between the beluntas leaf extract and the number of Streptococcus mutans bacteria, which will then continued by post-hoc test with the Tuckey method to find out which dataset is the most significantly different in among others.

Results: The beluntas leaf extract showed significant bacterial properties against Streptococcus mutans (P-Value = 0.00) which was expressed through definite MBC and MIC values. The results showed that the MIC and MBC values were 62.5 g/ml and 125 g/ml, respectively against Streptococcus mutans colonies.

Conclusion: The conclusion of this study was that the ethanol extract of the leaves of beluntas (Pluchea Indica L) showed antibacterial properties as indicated by the MIC value at a concentration of 62.5µg/ml and the MBC at a concentration of 125µg/ml against streptococcus mutans.

Keywords: Pluchea indica L., Minimum Bactericidal Concentration, Minimum Inhibitory Concentration, Streptococcus mutans
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Introduction

Dental caries is one of mouth and tooth health issues, it had become health problems throughout the world and had beed known to affect various bodily functions, such as mastication, food adsorption and digestion. It could also manifest into another systemic disease which stems from dental cavities developing further into infection sources.[1] The etiology of dental caries is primarily microorganisms, host (teeth and saliva), carbohydrate intake and time. Other predispository factors that contribute to the severity of caries are dental trauma and poor hygiene.[2] One of the main microbial agents responsible for dental caries is streptococcus mutans, the bacteria initiates carbohydrate fermentation process in dental cavities which in turn lowers the oral pH. The byproducts of this process are acids, which come in the form of lactate, acetate, formic and propionic acid. These acids will then start demineralizing enamel and dentin.[3,4]

Streptococcus mutans is a gram-positive, coccus-shaped bacteria, naturally present in human oral microbiota. This non-spore forming bacteria is also non-motile and facultatively anaerobic. Streptococcus mutans is also a cariogenic bacterium, which ferments carbohydrates and produces acid. This bacterium naturally lives in acidic environment and is able to stick into dental surface due to its ability to synthetize sticky extracellular polysaccharides. These extracellular polysaccharides mainly consisted of glucose polymers that also serves as further sticking area for another bacteria, which in turn will give way into formation of plaques.[5]

Streptococcus mutans produces Glucoxylltransferase (GTF) enzyme which can break down sucrose into soluble and insoluble extracellular polysaccharides, such as glucan and fructan that are closely related to plaque formation process and possessed strong cariogenic properties; these chemicals are also directly responsible for the virulence factor Streptococcus mutans.[6] Considering the 60 percent prevalence of dental caries among general population - which translates to 6 among 10 people, prevention should be prioritized as early as possible, the correct course would be to curtail the growth of Streptococcus mutans colonies.[7]

Mechanical means such as simply brushing one's teeth could be used to prevent excessive Streptococcus mutans growth, and chemically, over-the-counter mouthwash is also effective. Traditional

herbal medications are generally considered safer on some circumstances due to less side effects than modern, manufactured medication.[8] One of these herbal plants that could be used as preventive measure against Streptococcus mutans is Beluntas leaf (Pluchea indica l.) Beluntas leaf is an ubiquitous herb commonly found in Indonesia, it can grow easily along dry, hard and rocky surface, and can often be used as natural fence.[9] Nearly all parts of Beluntas plant can be used in various types of medication. Besides its obvious antibacterial agent, some parts even yield antidiabetic and antivenom properties.[10,11] Some chemical components that can be extracted from this plantare flavonoids, various essential oils, tannins, saponins and phenols. These chemicals alone could be used for various medicinal purposes, such as anti-expectorant and anti-bacterial agents, antimalarial drugs, antidiarrheals, remedies for various skin conditions and antipyretic drugs.[12]

Beluntas leaf also yields antibacterial agent in the form of flavonoid.[11,12] that could help disrupt bacterial trans-membrane proteins, which in turn starves bacterial cell of nutrition and prevents its growth, with the difference in polarity between the lipids that compose bacterial DNA and the alcohol groups in flavonoid compounds that can cause damage to the lipid structure of bacterial DNA so that bacteria lyses and dies.[13]

Material and Methods

This research is experimental using in vitro Broth Microdilution technique in laboratory setting and colony count by means of Total Plate Count method. Research samples used are as follows: (a) Streptococcus mutans ATCC 25175 culture obtained from Universitas Padjajaran Microbiology Laboratory; (b) 95% ethanol extract of Beluntas leaf (Pluchea indica l.) obtained from Balittro Bogor; (c) Repeat sample culture five times (Pentaplo). Methods used in this research are done in accordance of methods developed by CLSI (Clinical Laboratory Standard Institute) with specific modifications: addition of 2% sucrose solution on Mueller-Hinton Agar (MHA) media and Mueller-Hinton Broth (MHB).

MHA is a solid media specifically recommended by CLSI, clinically tested to provide good and consistent results on bacterial culture, MHB on the other hand is also a CLSI-recommended liquid media with similar good and consistent results on bacterial culture, the latter is crucial on the application of TPC (Total Plate Count).

Maceration is the preferred extraction method, which is described as preparing ingredients by 'marinating' it with ethanol solution. In preparing ethanol extract for stock solution, Beluntas leaf extract as many as 0.5 grams is scaled and mixed into a 1 ml 10% DMSO which will yield stock solution with a concentration of 500 µg/ml. Extracts used came in a pasta-like form. In preparation of Chlorhexidine positive control, as much as 600 µL of 0.2% of Chlorexidine were taken and diluted with a 600 µL MHB media into microtube and then subjected into vortex.

In preparing test subjects (microorganism), Streptococcus mutans would be bred in MHA media, be subjected to 2% sucrose addition, and will be incubated at 37°C and 5% CO_2 concentration for 24 hours. For bacterial inoculum preparation, mature Streptococcus mutans culture are taken as much as 2 times using ose needle, to suspended into NaCl (Merck KgaA, Germany). This bacterial suspension was measured using McFarland turbidity standard if it has reached 0.5 value in comparation to 1 x 10^8 and then will therefore be subjected to further dilution until its value reached 10^5.

In this research, there are eight ethanol extracts used with varying concentration: 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.3 µg/ml, 15.6 µg/ml, 7.8 µg/ml, 3.9 µg/ml and 1.95 µg/ml. Observation is then performed by observing total colonies on MHA media to determine MBC and MIC values using colony counter (FUNKE GERBER Colony Star 8500 Colony Counter, Germany). How to count Bactericidal Percentage:

$$\frac{\text{Bacterial Control Colony Mean} - \text{Bacterial Concentrated Colony Mean}}{\text{Bacterial Control Colony Mean}} \times 100\%$$

The following is how to count bacterial colony percentage (CFU/mL):

$$\frac{\text{Total Colony Count}}{\text{Colony Volume of in-Criteria Bacterial Sample}} \times \text{Dilution Factor}$$

Data analysis testing is performed to test normality using Kolmogorov-Smirnov test. If data is normally distributed, then One Way ANOVA parametric test will be performed, this test is designed to measure the significance between Beluntas leaf ethanol extracts against Streptococcus mutans colony. Post-Hoc test with Tuckey method is then performed to determine which dataset is the one most significantly different than the other.

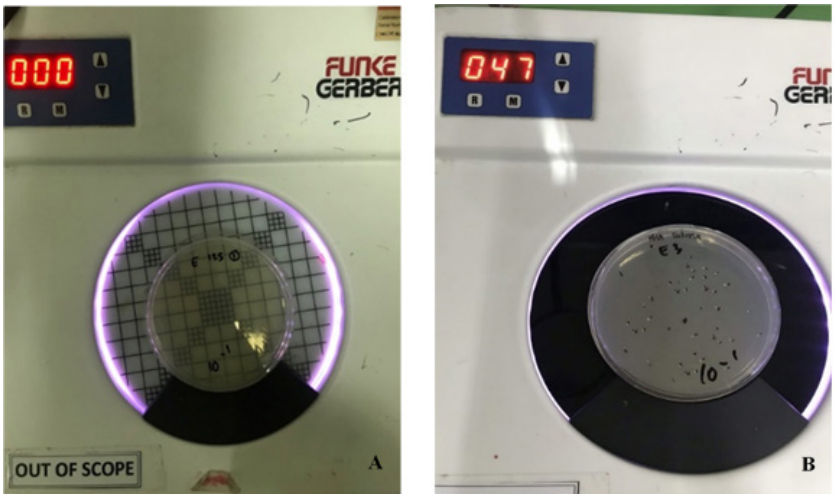
Results

Mean control colony value of streptococcus mutans is obtained from every addition when dilution processes of bacterial culture are performed **table 1**. Mean value of said control colony is measured at 25200 CFU/mL which will in turn be provided

Table 1. Streptococcus mutans Colony Count after 24-hour Incubation with multiple concentrations of Beluntas Leaf Ethanol Extract

Concentrations	Average Value of Colony (CFU/mL)	Average Bactericidal Efficiency	Notes
250 µg/mL	0	100%	
125 µg/mL	0	100%	MBC
62.5 µg/mL	828	96.7%	MIC
31.25 µg/mL	5388	78.5%	
15.62 µg/ mL	12700	49.5%	
7.8 µg/mL	15288	39.3%	
3.9 µg/mL	16863	33.1%	
1.95 µg/mL	17142	30.8%	

Figure 1. A. Total colony count of streptococcus mutans bacteria on beluntas leaf ethanol extract 125 µg/mL (MBC), B. Total colony count of streptococcus mutans pada Beluntas Leaf Ethanol Extract at 62.5 µg/mL (MIC)



further as reference point for determination of MBC and MIC value in this research.

Concentration of 125 µg/mL yields the biggest bactericidal efficiency against Streptococcus mutans, with colony count measured at 0 CFU/mL, Beluntas leaf ethanol extract also yields the smallest bactericidal efficiency against said microorganism at 19.5 µg/mL with colony count measured at 17142 CFU/mL.

Chlorhexidine on the other hand, yields maximum bactericidal efficiency against Streptococcus mutans at a concentration of 1.25 x 10-2% with 0 CFU/mL, its minimum bactericidal efficiency against streptococcus mutans is at 7.81 x 10-4% with colony count measured at 176 CFU/mL.

MIC value of Beluntas leaf ethanol extract is located at 62.5 µg/mL concentration with bactericidal efficiency measured at 96.7% and its MBC is measured at 125 µg/mL with 100% kill efficiency.

Statistical testing results of this research is obtained using One Way Anova method with P-Value set at 0.00, results then showed lower p value ($p < 0.05$) which signifies significant bactericidal properties on Beluntas leaf extract against Streptococcus mutans colony, which also showed in its MIC and MBC values. Another Post-Hoc Test with Tuckey method is also needed if One Way Anova test results yield significant value, the former is needed to determine which dataset is the most significantly relevant against the others.

Post-Hoc Tuckey test results showed significant mean difference on 26 comparisons of Beluntas leaf ethanol extracts (Pluchea indica L.) against mutans streptococci. Mean Difference column showed that nearly all pairs' P-values differed significantly.

Discussion

Beluntas leaf ethanol extract has shown significant inhibitory and bactericidal properties against Streptococcus mutans growth, with MIC value determined at 62.5 µg/mL with bactericidal/kill efficiency at 96.7 percent and MBC determined at 125 µg/mL with efficiency of 100 percent. Chlorhexidine yields MBC value at 1.25 x 10^{-2} % with 100% efficiency, and MIC value determined at 7.81 x 10^{-4} % with 99.7 percent efficiency. Statistical test results with P-value set at 0.00 showed that MIC and MBC values obtained have significant effects in hampering the growth of streptococcus mutans.

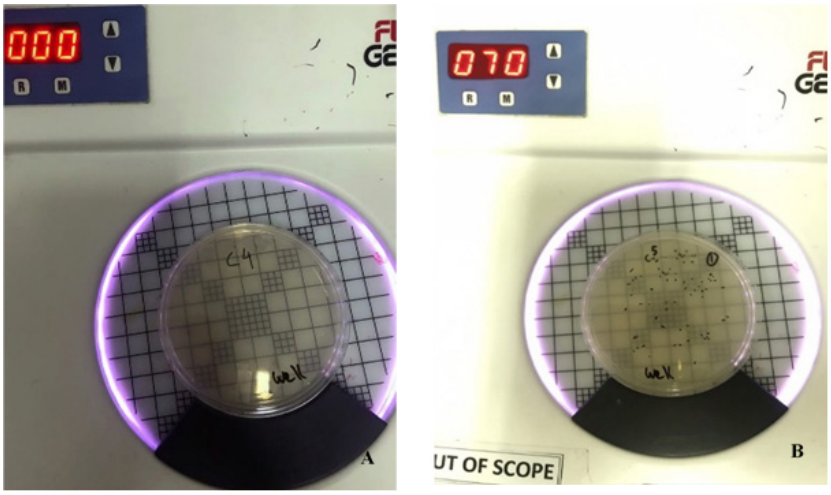
These results are consistent with research by Annisa, et. al.(2019)[11] where Effectiveness of less pluchea indica (L.) beluntas leaf ethanol extract on saliva bacteria in vitro. Research done by Dewa, et. al. (2020) had also shown similar results against growth of *Streptococcus Pyogenes ATCC 19615*.[14]

Bactericidal properties of Beluntas leaf against Streptococcus mutans as shown with its MBC and MIC values could be explained by its inherent

Table 2. Total colony count of streptococcus mutans after 24-hour incubation on different chlorhexidine concentrations

Chlorhexidine Concentration	Mean Colony (CFU/mL)	Percentage Kill Efficiency	Notes
0,1%	0	100%	
0,05%	0	100%	
0,025%	0	100%	
1,25 x 10^{-2} %	0	100%	MBC
6,25 x 10^{-3} %	57	99,7%	MIC
3,12 x 10^{-3}%	142	99,4%	
1,56 x 10^{-3} %	153	99,3%	
7,81 x 10^{-4} %	176	99,2%	

Figure 2. A. Streptococcus mutans total colony count on chlorhexidine concentration 1.25 x 10^{-2}% (MBC), B. Streptococcus mutans total colony count on chlorhexidine concentration 6.25 x 10^{-3}% (MIC)



anti-microbial properties.[15] Previous phytochemical studies have indicated the presence of secondary metabolites such as phenolics, flavonoids, terpenoids, tannins and saponins.[16,17]

Flavonoids are secondary metabolites of polyphenols with significant antibacterial properties, these antibacterial agents could interact directly with bacterial DNA and destroy them. This interaction could be elaborated as follows: the chemical substance will form complex molecules with extracellular proteins, which in turn will inhibit bacterial cytoplasmic functions by means of reducing the fluidity of inner and outer membrane of bacterial cells, microsome and lysosome. In the end, substrate sticking process will be halted because by then cell membrane have been made non-functional, which in tun would lead to bacteriolysis and eventual death.[18,19,20]

Other substance, saponin, which is one of many secondary metabolites of the plants, marked by formation of stable foam when diluted with water. This compound is a form of glycoside that also yield sugary molecules with two types of aglycon, steroid and triterpenoid.[21] Saponin has lots of biochemical properties, one of them is antibacterial agent. Saponin does this by causing bacterial cell membrane leakage which causes disruption in membrane permeability that will in turn severely impair bacterial life cycle. Saponin will bind against cytoplasmic membrane through its outermost layer and destabilize membrane integrity, this will cause certain bacteriolysis and eventual death to the organism.[22,23]

Tannins are grouped into polyphenols and are soluble in water, they are also one of the most abundant soluble compounds in plants. Tannins induce antibacterial properties by utilizing its phenyl ring, which is inherently antiseptic. Tannins do this by forming complex compounds with proteins through hydrogen bond, which is when formed will denaturizes bacterial cell and renders cell metabolism disrupted.[24] Tannins are also capable of deactivating adhesin (a type of molecules that enable bacteria to latch onto host cells, found on cell surface) and disrupting protein transport on inner cell layers which will in turn starves bacterium off protein, causes disintegration on cell walls and incite bacteriolysis and eventual death.[25,26]

Phenol is a secondary metabolite byproduct of plants, phenol also has pronounced antibacterial properties, in which it severs peptidoglycan cross-bond off in the process of entering cell membrane. It will then trigger leakage of proteins and phospholipids out of cell and deprive bacterium from nutrition as well as increasing the membrane permeability in the process. Specific enzyme biosynthesis inside the cell will also be impaired and this damaged metabolism will render bacteria unable to sustain life in the end.[27,28]

Terpenoid, which is group of organic chemicals consisted of few isoprene units, is naturally found in plants and acted as part of defense mechanism against external threats. Terpenoids are usually soluble in fat and are located within plant cell cytoplasm.[29,30] The antibacterial properties of terpenoids revolve around their capability to react against cell surface on the bacterium, create a strong polymer bond and disintegrate cellular wall in the process. This process will reduce the permeability of cellular wall and will duly impair its metabolism, therefore forcing it into bacteriolysis and eventual death.[31,32]

Conclusion

Ethanol extract of Beluntas leaf could be developed

and utilized as natural anti-bacterial agent against Streptococcus mutans, significant MIC and MBC values are measured at 62.5 µg/mL 125 µg/mL respectively with colony count measured at 0 CFU/mL.

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Conflict of Interest

The authors report no conflict of interest.

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