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Original Research Article

Evaluation of minimum inhibitory concentration of methanol extract of cacao seeds on growth of *Streptococcus mutans* bacteria by microdilution method

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ABSTRACT

Background: Indonesia is famous for its medicinal plants. Cocoa bean (*Theobroma cacao* L.) is a plant widely used as a traditional medicine alternative to cure diseases. Cocoa beans contain alkaloids, flavonoids, saponins and tannins which are antibacterial and have potential as antioxidants. Various dental and oral diseases that occur are caused by bacteria, one of which is *Streptococcus mutans* which causes dental caries. Bacteria that play an important role in the occurrence of dental caries is *Streptococcus mutans*. Dental caries can be prevented by chemically and mechanically maintaining oral hygiene using antiseptics that have antibacterial properties. This study aimed to test the minimum inhibitory concentration of methanol extract of cocoa seeds on the growth of *Streptococcus mutans* with the microdilution method.

Methods: The research method is the microdilution method with concentrations of 0.753%, 1.563%, 3.125%, 6.25%, 12.5%, 25%, 50%, 100%. *Streptococcus mutans* bacteria were obtained from the University of Indonesia's veterinary laboratory.

Results: The results showed that the minimum inhibitory concentration of methanol extract of cocoa beans on the growth of *Streptococcus mutans* was at a concentration of 1.563%.

Conclusions: In conclusion, the methanol extract of cocoa beans has an inhibitory effect on *Streptococcus mutans* bacteria and there is a minimum inhibitory concentration at a concentration of 1.563%. It is recommended to continue to toothpaste medication and do preclinical trials.

Keywords: Mouth washing, Laban leaves, Steeping water, Plaque accumulation

INTRODUCTION

Indonesia is famous for its wealth of medicinal plants. Traditional medicine is an alternative treatment Indonesian people use for generations.¹ One of the natural ingredients used as conventional medicine is cocoa bean (*Theobroma*

cacao L.).² Cocoa beans have active components of polyphenols which have activity in preventing degenerative infectious diseases and oral diseases which can reduce the formation of biofilms and acid products from *Streptococcus mutans* which causes dental caries.³ Cocoa bean extract has antioxidant activity and with an

IC50 value of 170 ppm and is effective in inhibiting the growth of mutant *Streptococcus* bacteria.⁴ Preliminary test results of methanol extract of cocoa beans have inhibition against *Streptococcus mutans* at a concentration of 10% to 80%. Various bacteria are present in the oral cavity, but only a few bacteria cause dental caries, including *Streptococcus mutans*.⁵ Dental plaque or biofilm plays an important role in dental caries.^{6,7} Biofilm is a collection of microorganism cells attached to the tooth surface and covered by a carbohydrate adhesive secreted by bacteria. Therefore, it is necessary to use medicinal ingredients to control bacteria.⁸

Dental and mouth disease in Indonesia is still very high 63%, Aceh province has dental problems 47.0%, children 92.6%, and parents 96.2%.^{9,10} So it is necessary to inhibit the growth of bacteria.^{11,12} Even though toothpaste contains active anti-caries ingredients, dental caries is still high. If fluoride and triclosan are increased, fluorosis, toxicity and tooth demineralization will occur. This is what causes concern about the use of herbal toothpaste base ingredients.¹³ The objective of this study is to test the minimum inhibitory concentration (MIC) of methanol extract of cocoa seeds on the growth of *Streptococcus mutans* with the microdilution method.

METHODS

The research design was a laboratory experiment with a post-test only control group design. To determine the minimum inhibitory concentration of methanol extract of cocoa beans on the growth of *Streptococcus mutans* with the microdilution method. The sample used in this study was cocoa bean extract from Sare Aceh, *Streptococcus* Bacteria. *mutans* from the faculty of veterinary medicine microbiology laboratory at the Universitas Syiah Kuala.

The change measured or observed for the phytochemical test is a change in color and for the MIC it is seen from the turbidity if it is cloudy, it indicates the bacteria are not inhibiting, if it is clear, it indicates the presence of bacterial inhibitory activity. If life is marked with turbidity which means positive (+), if there is inhibition it is marked with a clear color which means there is inhibition it is marked with negative (-).¹⁴ Phytochemical test in the faculty of mathematics and natural sciences laboratory, Shiakuala University, Banda Aceh. antibacterial activity test, MIC was carried out at the microbiology laboratory, Faculty of Veterinary Medicine (FKH), Syiah Kuala University, Banda Aceh, April to June 2022. Research locations for making extracts located at the USK Agriculture laboratory in April 2022, and MIC testing at the USK Faculty of Veterinary Medicine laboratory, May 2022 to June 2022. The research time is March 2022 to August 2022.

Research procedure

Preparation of tools and materials: The tools used are jars, mixing spoons, filter paper, glass funnels, rotary evaporators, spirit lamps, petri dishes, test tubes, digital

scales. The materials used are extracts of laban leaves, mutan streptococcus bacteria, distilled water. Sample preparation of cocoa beans: Random sampling of 2.5 kg of cocoa beans. The cocoa beans are air-dried at room temperature for 3 weeks. Seeds that are already dry in a blender become simplicia. Simplicia was macerated with methanol solvent 3x24 hours. The filtrate was filtered and then carried out by a rotary evaporator to obtain a thick methanol extract of cocoa beans.

Evaluation the secondary metabolites of cocoa beans: Evaluation for alkaloid compounds. 1 g of dry sample of cocoa beans was ground, added with 1 ml of concentrated ammonia, crushed and filtered. 10 mL of 0.5 N hydrochloric acid was added, shaken vigorously, allowed to stand until it separated. The hydrochloric acid layer was taken and divided into three tubes and each tube was tested for the presence of alkaloids. The addition of Mayer's reagent will cause a white precipitate, while Dragendorff's reagent will cause a reddish precipitate, Wagner's reagent will cause a brown precipitate, indicating a positive presence of alkaloid compounds. Test Steroid, terpenoid and saponin compounds, 10 g of dry sample of cocoa beans added to distilled water and shaken vigorously. The presence of stable foam for 30 minutes indicated the presence of saponins, the solution was hydrolyzed with HCl and tested with Liebermann-Burchard reagent. Green or blue indicates the presence of steroid saponins and red indicates the presence of triterpenoid saponins.

Test for Flavonoid compounds, 10 g of dried guava leaves were mashed, add 10 mL of 80% ethanol, then 0.5 g of magnesium metal added with 0.5 M HCl. A pink or purple color indicates the presence of flavonoids. MIC with the microdilution method. The management of the test bacteria used *Streptococcus mutans* from the laboratory of the faculty of veterinary medicine, Universitas Syiah Kuala, which was rejuvenated first from the main culture. Bacteria are rejuvenated by growing the bacteria in liquid BHI (brain heart infusion) media which is incubated for 48 hours at 37°C. The test bacterial suspension was standardized with 0.5 McFarland solution so that the total density of bacteria used was the same as 1.5×10^8 CFU/mL, then gram staining was performed. MIC by preparing a microplate consisting of 96 wells (8 rows and 12 columns). Wells 1,2,3,4,5,6,7,8 in the first row are filled with 150 µl BHI (brain heart infusion) liquid as a medium. Then 150 µl of 100% (1 g/ml) cocoa bean extract was taken and put into wells 1, 2, 3, 4, 5, 6, 7, 8 in line 1 and serial dilution was carried out, dilution results 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.563%, 0.753%. Then in all wells 1, 2, 3, 4, 5, 6, 7, 8 were added to the bacterial suspension of *S. mutans*, 10 µ each. Then the microplates were incubated facultatively anaerobically in a desiccator at 37°C for 48 hours. The MIC was determined based on the turbidity of the culture and the reading of the incubation results with the minimum inhibitory concentration value being the smallest concentration which gives a negative result. Inserted into the indicator with a temperature of 37°C for 48 hours, you will see bacterial colonies indicating the

presence of bacterial growth from MHA which remains sterile or no colonies indicating no bacteria. KBM was determined with the smallest concentration which showed no growth of *Streptococcus*.¹⁵ The test was carried out 3 repetitions.¹⁶

RESULTS

Collecting a sample of 2.5 kg of cocoa beans, then washed and air-dried at room temperature for 3 weeks. Samples of dried cocoa beans were blended until smooth to become simplicia.¹⁷

Table 1: Phytochemical test results of methanol extract of cocoa beans.

Secondary metabolite	Cocoa bean methanol extract	Description
Phenolic	+	Formed in green
Tannins	+	Formed cloudy white
Flavonoids	+	Formed pink/purple color
Steroids	-	No green/bluish color is formed
Terpenoids	+	Formed red
Saponins	+	Formed bubbles or foam
Alkaloids		
dd	+	Formed brick red color
Mayer	+	A white precipitate formed
Wakner	+	A brown precipitate formed

Further extraction by maceration using 90% methanol solvent 3x24 hours, the filtrate obtained is carried out by a Rotary evaporator. Phytochemical test of cocoa beans to determine secondary metabolite compounds contained in a plant. Phytochemical tests include tests for alkaloids, steroids, terpenoids, saponins, flavonoids and phenolics. Alkaloid test can be done by adding Mayer, Dragendorff and Wagner reagents. The addition of Mayer's reagent formed a white precipitate indicating a positive presence of alkaloids, while the addition of dragendorff showed a red precipitate and the addition of Wagner's reagent showed a positive result if a brown precipitate formed. Phytochemical tests for steroid and terpenoid compounds can be carried out using the Liebermann-Burchard reagent and if the color changes to red it indicates positive for terpenoids, whereas if the color turns green or blue it indicates the presence of steroid compounds. The phytochemical test of flavonoid compounds can be carried out by extracting the residue with ethanol and adding HCl and Mg powder to produce a pink or purple color indicating the presence of flavonoid compounds.¹² The results of the phytochemical test of the methanol extract of Sare Aceh cocoa beans contained secondary metabolites, including, phenolics, tannins, flavonoids, terpenoids, saponins, and alkaloids. The minimum inhibitory concentration is the smallest concentration that gives the first negative sign. Determination of minimum inhibitory

concentration was carried out on mutant *Streptococcus* bacteria. The chosen method is the microdilution method. Cocoa bean extract at a concentration of 1.563, there is a first negative value, which means that inhibition of bacteria begins, so it can be concluded that the minimum inhibitory concentration value is at a concentration of 1.563 (Table 2).

Table 2: Minimum inhibitory concentration.

Concentration (%)	MIC		
	I	II	III
100	-	-	-
50	-	-	-
25	-	-	-
12.5	-	-	-
6.25	-	-	-
3.125	-	-	-
1.563	-	-	-
0.781	+	+	+
Aqua	+	+	+

There is bacterial growth (+), No bacterial growth (-)

DISCUSSION

Phytochemical tests on cocoa beans included tests for alkaloids, steroids, terpenoids, saponins, flavonoids and phenolics. Alkaloid test can be done by adding Mayer, Dragendorff and Wagner reagents. The addition of Mayer's reagent formed a white precipitate indicating a positive presence of alkaloids, while the addition of dragendorff showed a red precipitate and the addition of Wagner's reagent showed a positive result if a brown precipitate formed. Phytochemical tests for steroid and terpenoid compounds can be carried out using the Liebermann-Burchard reagent and if the color changes to red it indicates positive for terpenoids, whereas if the color turns green or blue it indicates the presence of steroid compounds. The phytochemical test of flavonoid compounds can be carried out by extracting the residue with ethanol and adding HCl and Mg powder to produce a pink or purple color indicating the presence of flavonoid compounds.¹⁴⁻¹⁶

The results of the phytochemical test of cocoa beans contain secondary metabolic compounds of terpenoids, steroids, saponins, flavonoids and phenolics. The presence of terpenoids together with steroids is very common in plants, because biosynthetically, steroid compounds are derived from terpenoid compounds. The results of the antibacterial test of the methanol extract of cocoa beans against *Streptococcus mutans* showed that the methanol extract of cocoa beans had antibacterial inhibitory activity. The minimum inhibitory concentration value is the smallest concentration, giving a negative result. The results showed that the calculation of the percentage of bacterial inhibition was found at the minimum inhibitory concentration of 1.563. The inhibition in the growth of *Streptococcus mutans* bacteria is thought to be due to the presence of compounds in the methanol extract of cocoa

beans such as compounds, alkaloids, flavonoids, tannins, saponins, polyphenols which can cause bacterial death. Alkaloids can be antibacterial by interfering with the constituent components of peptidoglycan in bacterial cells, causing the cell wall layer to not form completely and causing cell death. Phenol alkaloid compounds are anti-toxic compounds that cause the three-dimensional structure of bacterial proteins to be disrupted and open into random structures, which can cause protein denaturation and biological activity to be damaged so that the growth of *Streptococcus mutans* stops.¹⁷ Flavonoids function as antibacterial by forming complex compounds against extracellular proteins that cause damage to the structure and changes in cell membrane mechanisms. Flavonoids are phenolic compounds that inhibit the synthesis of nucleic acids from bacteria and inhibit the motility of bacteria.¹⁸ Methanol extract of cocoa beans also contains saponins, which act as strong surfactant agents like soap, because they can reduce cell surface tension. Saponins can inhibit bacterial growth because they can reduce the surface tension of the bacterial cell wall, when interacting with the bacterial cell wall it will rupture or lyse. When saponins interfere with the surface tension of the bacterial cell wall, antibacterial substances will enter the cell and interfere with metabolism so that the bacteria will die. Saponins that are absorbed on the cell surface will cause damage by increasing membrane permeability, so that the essential ingredients needed by bacteria are lost, causing cell death. Polyphenols have the potential as an antibacterial by poisoning protoplasm, damaging bacterial cells. Tannins in the methanol extract of cocoa beans can shrink cell walls or cell membranes, causing disruption of cell permeability. As a result of disruption of permeability causes the cell to die.

CONCLUSION

Cocoa bean methanol extract can inhibit mutant *Streptococcus* bacteria MIC at a concentration of 1.563%.

Recommendations

Further research is expected to explore an antibacterial test of cocoa bean methanol extract toothpaste on the growth of mutant *Streptococcus* bacteria. For further research, cocoa beans' methanol extract can be considered an ingredient in toothpaste.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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