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ANTIBACTERIAL ACTIVITY AND INHIBITION OF BIOFILM OF *Hibiscus tiliaceus* STEM BARK METHANOL EXTRACT TO *Streptococcus mutans* GROWTH

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ABSTRACT

Dental caries is an infectious disease that occurs in the oral cavity preceded by the formation of dental plaque or biofilm. This study aims to examine the inhibition and biofilm inhibition of hibiscus tree bark extract (Hibiscus tiliaceus). The test methods used were Minimum Inhibitory Concentration (MIC) and Minimum Killing Concentration (MBC) while the analysis of biofilm inhibition used UV-VIS spectrophotometry at a wavelength of 580 nm with McFarlandII standard (6x108 CFU/ml). The results showed that the methanol extract of hibiscus bark at a small concentration of 0.5% could inhibit and kill Streptococcus mutans as indicated by the number of colonies that grew less with a value of 3.34 x 107 CFU/ml in colonies that grew at an extract concentration of 2. % and 1%. Biofilm inhibition is shown by the decreasing absorbance value due to the increased extract concentration so that less biofilm formation occurs.

Keywords: *biofilm, inhibition, dental plaque, Streptococcus mutans.*

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INTRODUCTION

Dental caries is a common infectious disease that often occurs among the public, initiated by the formation of dental plaque or biofilm in the oral cavity (Dewi, 2015). Biofilm is a collection of microbial cells, especially bacteria, which are firmly attached to a surface accompanied by organic materials and covered by an extracellular polymeric matrix secreted by bacteria (Madigan, 2014). Plaque control is the removal of microbial plaque and prevention of its accumulation on the tooth surface and its surroundings (Eke, 2012). Streptococcus mutans is a gram-positive facultative anaerobic bacterium that can produce lactic acid as part of its metabolism and is useful for the life of these bacteria. Streptococcus mutans has the ability to bind sucrose to the tooth surface by forming water-insoluble glucans and polysaccharides which help in binding the bacteria to the teeth (Simon, 2007). Streptococcus mutans can lower or maintain the pH of the mouth at a moderately acidic value, leading to conditions that are favorable for its own metabolism and unfavorable for other coexisting species. The decrease in pH caused by Streptoccocus mutans can ferment

sugar into acid. This acid attaches to tooth enamel which causes demineralization of tooth tissue and cavities in teeth (Putri, 2017).

Streptococcus mutans is a normal flora in the oral cavity which can turn into a pathogen if there is an excessive increase in the number of colonies. In dentistry, Streptococcus mutans plays an important role in the formation of caries. Prevention of caries and periodontal disease by improving dental health has become a major goal in the world of dentistry since it is known that dental plaque is the dominating factor causing tooth loss |(Metwalli, 2013). Biofilms are groups or communities of microorganisms that have attached themselves to a surface in a moist environment and are found in an environment with sufficient nutrient flow. Biofilm growth goes through the following stages: bacterial attachment to the substrate, bacterial growth, bacterial cell division, colonization in the surrounding environment and biofilm formation. These bacteria do not work individually to form biofilms, but gather into long chains to help initiate the initial stages of biofilm formation. Mature biofilms are complex heterogeneous structures in dormant and actively growing bacterial colonies with enzymes, excreted products, and small ducts forming part of the entire structure. In some cases it will form a pillar-like structure (Armitage, 2005). Dental plaque is formed by involving fermentation carried out by the main bacteria that causes caries, namely Streptococcus mutans which is capable of producing glucosyl transferase (GTF) which can convert sucrose into glucan and then form dental plaque (Shu, 2013). Plaque can be removed by mechanical cleaning and chemical inhibition [10]. Mechanical cleaning can be done by brushing your teeth, while chemical cleaning can be done with mouthwash. One of the goals of brushing your teeth is to inhibit the growth of plaque bacteria (Diah, 2012).

The use of traditional medicine is generally considered safer because it has relatively few side effects compared to modern medicine (Raina, 2011). Several plants are known to be used as ingredients for the prevention and treatment of dental and oral diseases including fresh tea leaf extract (Dyah, 2011), clove flower extract (Nurhayati, 2017), turmeric extract (Nyoman, 2019). Apart from that, there are also hibiscus plants which belong to the Magnoliopsida class which are known to the public only as wild plants, only left as they are so that their use as medicine is still lacking. However, this plant has many benefits as a traditional medicine, cooling for fever, helping hair growth, cough medicine, bloody/mucous diarrhea and tonsils. People in Ambalau District, South Buru Regency, Maluku use the bark of the hibiscus tree as a medicine for toothache. In the treatment, the bark of the hibiscus tree is scraped sufficiently and then placed on the aching tooth. In the city of Ambon, this plant is widely found along the coast of the Leihitu Peninsula. Phytochemical tests were carried out to evaluate the pharmacological effects of the ethanol extract of the leaves and bark of this plant for cytotoxic, antibacterial, analgesic and neuropharmacological activities. The antibacterial test of H. tiliaceus leaf extract did not show antibacterial activity while the bark extract showed inhibitory activity against Staphylococcus aureus and Staphylococcus epidermidis (Awal, 2016). The content of flavonoids in H. tiliaceus inhibits the process of glucosyltransferase activity which plays an important role in the formation of dental caries, while tannins deactivate glucosyltransferase enzymes and adhesion molecules (surface proteins on bacterial cells) (Kumar, 2013).

Mechanism can be caused by the accumulation of lipophilic components found in the cell wall or cell membrane, causing changes in the composition of the cell wall, the antibacterial action of each secondary metabolite compound is different. Secondary metabolite compounds inhibit bacterial growth starting with damaging the cell wall. Flavonoid compounds can penetrate peptidoglycan which is polar because flavonoids are also polar, while on the other hand phenolic compounds damage the bacterial wall by breaking the peptidoglycan bond, in gram-positive bacteria the cell wall contains 90% peptidoglycan and a thin layer of teichoic acid and teichoic acid which are negatively charged (Mardigan, 2014). The mechanism of inhibition of bacteria by phenolic compounds can interfere with the peptidoglycan component of bacterial cells, so that the cell layer is not completely formed. Alkaloid compounds work by inhibiting cell wall synthesis (Lamothe, 2009). Until now, research on the test of waru tree bark extract as an antibacterial agent for dental caries has never been carried out. Based on empirical data obtained from the Ambalau community in South Buru Regency who use waru tree bark to treat aching teeth, a comprehensive study was carried out on antibacterial activity and biofilm inhibition of waru tree bark extract on the growth of Streptococcus mutans bacteria.

METHODE

Tools and materials

The tools used are maceration tools, autoclaves, petri dishes, erlenmeyer, measuring cups, beakers, incubators, incubator shakers, spirit lamps, Laminar Air Flow (LAF), refrigerators, round loops, ovens, tweezers, knives, rotary evaporators, test tube, analytical balance, micro pipette, hot plate, UV-VIS spectrophotometry, colony counter. The materials used were waru tree bark, methanol, Nutrient Agar (NA), aluminum foil, Nutrient Broth (NB), NaCl, DMSO, Tween 80, Listerine citrus, tissue, and Streptococcus mutans bacteria.

Procedure

The research was carried out in several stages, namely sampling, preparation of methanol extract of waru tree bark, preparation of media, rejuvenation of bacteria. Sampling was carried out by selecting mature trees and without comparing them with other areas. The hibiscus tree is felled, the stem is skinned, cut into small pieces and dried. Extraction was carried out by maceration, as much as 500 g of simplicia was macerated with 96% methanol solvent until all parts of the simplicia were covered with solvent, stirred and precipitated for 4 days, then filtered to obtain filtrate. The filtrate was concentrated with a rotary evaporator at 50ºC to obtain a thick extract of 250 g. Rejuvenation of bacteria obtained from the laboratory of the Biology Education Study Program, Muhammadiyah University, Purwokerto, was carried out in a slanted Nutrien Agar medium, incubated at 37ºC for 1x24 hours. Furthermore, the test culture derived from pure culture, 1 ose is taken and then inoculated by streaking it on the Nutrien Agar medium, then incubated at 37ºC for 1x24 hours and the bacterial absorption is measured by UV-VIS spectrophotometry at a wavelength of 580 nm.

Antimicrobial evaluation

1) MIC Testing (Minimum Inhibitory Concentration)

As much as 2 g of hibiscus bark extract was added with 0.2 ml of DMSO as a solvent, then added 9 ml of distilled water using a micropipette, a stock solution of 2% methanol extract of waru tree bark was made. The MIC test was carried out by preparing five concentrations of the methanol extract of waru tree bark, namely 2%; 1%; 0.5%; 0.25% and 0.125%, each concentration was made up to 10 ml in size, then added 0.1 ml of Streptococcus mutans bacteria, incubated in an incubator shaker for 1x24 hours at 37 ºC, 280 RPM (so that oxygen dissolves more) and observed the level of turbidity at a wavelength of 580 nm.

2) Testing of KBM (Minimum Kill Concentration)

10 ml of NA medium was put into a petri dish and then allowed to solidify. Further dilution (the number of bacterial cells is diluted from 10-2, 10-4, 10-6 to anticipate if bacterial cell growth accumulates so that it is diluted to 10-6) serial levels in NaCl media which have been put 1 ml in 9 ml NaCl and 0.1 ml of test bacteria in 9.9 ml NaCl. The bacteria that have been added to the extract and the bacteria are spread on the media for the MIC test. The results of the MIC test were taken 100 µl and then spread on NA media, then incubated for 1×24 hours at 37 ºC and counted the number of bacterial colonies that grew. The KBM value is indicated by the absence of microbial growth at the lowest concentration.

Biofilm inhibition

Biofilm inhibition was carried out using the microdilution method. Suspension of bacteria was made in Nutrient Broth (NB) medium and McFarlandII standard (6x108 CFU/ml) was used. Serial dilutions were then carried out to contain each hibiscus bark extract, namely 0.18% w/v; 0.091% w/v; and 0.023% w/v with Dimethyl Sulfoxide (DMSO) as solvent. The positive controls used Listerine citrus, and tween 80 as plaque controls. A total of 3 ml of the test solution was placed in the tube and the test was carried out on 1 test tube and 1 blank tube.

1) The test tube contains the test extract solution with the addition of 10% v/v bacterial suspension, while the blank tube contains the extract solution with the addition of 10% v/v saline solution without adding the bacterial suspension. The tube is inserted into the spectrophotometer after which it is incubated for 18-24 hours at an incubator temperature of 36.6 °C.

2) The tube is removed from the incubator then the test solution is discarded and washed with running water 3 times and dried.

3) Each tube was added with 1% crystal violet dye in 125 µl of distilled water and left for 15 minutes, then washed again with running water 3 times and left for 15 minutes. Then 200 µl of 96% ethanol was added and allowed to stand for 15 minutes.

4) The test results in the form of optical density (OD) are read with a spectrophotometer at a wavelength of 580 nm.

DISCUSSION RESULT

MIC test of methanol extract of hibiscus stem bark against bacteria Streptococcus mutan

Preliminary test results for determining the minimum inhibitory concentration (MIC) of the methanol extract of hibiscus bark using the Erlenmeyer dilution method serially. Some treatment concentration of 2%; 1%; 0.5%; 0.25% which was added to the bacterial suspension of Streptococcus mutans and methanol extract of waru tree bark after being inoculated in an incubator shaker for 1x24 hours with a temperature of 37^0C, 280 RPM and compared with the antibiotic Ciprofloxacin as a control (+), media control as a negative control , the turbidity of the test sample was observed (Table 1).

Table 1. Turbidity levels of Nutrien Broth media for Streptococcus mutans bacteria at various concentrations of hibiscus bark extract

Concentration	**Turbidity of Bacterial Growth Media Streptococcus mutans**
2%	cloudy / bacteria growth
1%	cloudy / bacteria growth
0,5%	cloudy / bacteria growth
0,25%	cloudy / bacteria growth
Control (+)	Clear / not growth bacteria
Control (-)	cloudy / bacteria growth

The results of this inhibition are difficult to evaluate because the results of dilution by Erlenmeyer dilution at a concentration of 2% − 0.25% show the same level of turbidity. This is due to the basic color of the methanol extract of the bark of the hibiscus tree which is dark brown. So to find out the effect of giving concentrations of hibiscus bark extract on Streptococcus mutans bacteria, the spread method was carried out on Nutrien Agar media by adding 0.1 ml of the dilution results that had been diluted in NaCl media. Whereas in the control (-) and control (+) 0.1 ml was taken to spread on Nutrien Agar media. According to Taslihan (1986) that a cloudy medium means that bacteria can still grow so that antibiotics are not effective, whereas if the medium is clear it means that antibiotics are effective in inhibiting bacterial growth.

Biofilm inhibition test from methanol extract of waru tree bark

The biofilm inhibition test was carried out at several concentrations of the extract and control using the tube dilution method by measuring the absorbance at each concentration and the treated control was measured absorbance using UV-VIS spectrophotometry with a wavelength of 580 nm. The results of measuring the absorbance or OD (Optical Density) of each concentration in the biofilm inhibition test using several extract concentrations and two positive controls can be seen in Figure 2. The OD value at a concentration of 0.18% w/v was lower than an extract concentration of 0.023% w/ v and 0.091% w/v, this shows that the higher the concentration of the extract, the smaller the formation of Streptococcus mutans bacterial biofilms.

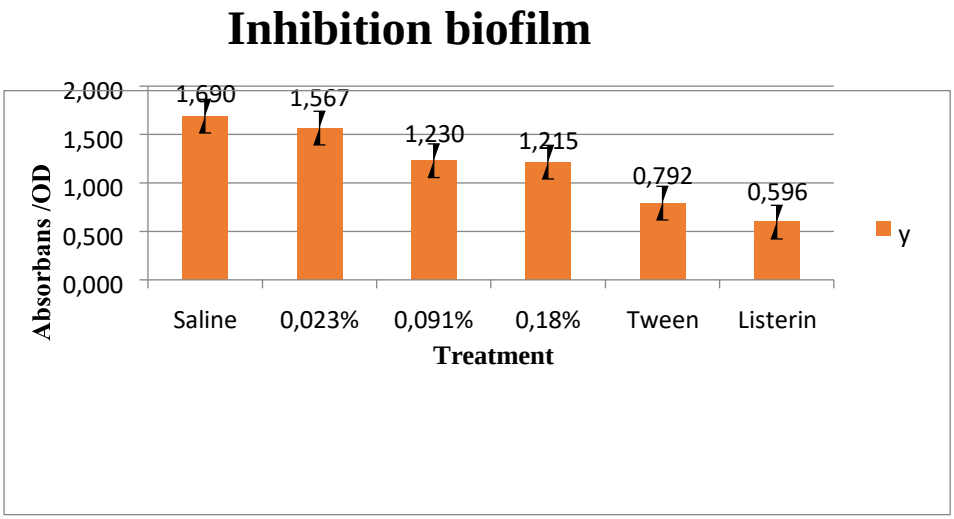

Figure 2. Results of the biofilm inhibition test of the methanol extract of waru tree bark

Based on the research results, it is known that the methanol extract of waru tree bark is very influential as an antibacterial against Streptococcus mutans with the presence of turbidity level at the Minimum Inhibitory Concentration (MIC) and the number of bacterial colony growth at the Minimum Killing Concentration (KBM) due to an increase in the concentration of methanol extract of the stem bark. hibiscus tree. MIC on 1% Streptococcus mutans bacteria, while MBC on 0.5% Streptococcus mutans bacteria which is indicated by a decrease in bacterial colony growth of 50% from sub-cultures. Antibacterial effects of other plant extracts have also been carried out including binahong leaf extract (Anredera cordifolia (Ten) Steenis) against Staphylococcus aureus and Pseudomonas aeruginosa. The increased concentration of binahong leaf extract in the MIC test for Staphylococcus aureus bacteria at a concentration of 25%, while for Pseudomonas aeruginosa bacteria at a concentration of 50%. Staphylococcus aureus at a concentration of 50%, while for Pseudomonas aeruginosa at a concentration of 100%, the growth of bacteria decreased by 99.9% from the inoculum from the culture (1). In addition, there is a citronella plant (Cymbopogon nardus L.) which can inhibit Streptococcus mutans bacteria in the 90% MIC test, an extract concentration of 0.18% was shown to have a value of 108.36% while biofilm inhibition activity at IC50 obtained a value of 0.137% (1).

CONCLUTION

The minimum inhibitory concentration of methanol extract of hibiscus bark at concentrations of 2%, 1% and 0.5% contained turbidity in the Nutrien Broth medium indicating that there was bacteria growing. Whereas in the follow-up test the minimum killing concentration (KBM) showed antibacterial activity from the methanol extract of hibiscus bark against Streptococcus mutans bacteria, the KBM value was indicated by a decrease in the number of colonies at a concentration of 0.5%. The biofilm inhibition test with a concentration of 0.18% w/v was able to inhibit biofilm formation with an OD value of 1.215 which showed that the lower the absorbance value, the smaller the biofilm formation.

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