Inhibition of cocoa pod husk (Theobroma cacao L.) extract gel against staphylococcus aureus

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Abstract

**Objective:** The aim of this study was to determined the inhibition zone of cocoa pod husk (Theobroma cacao L.) ethanol extract gel on staphylococcus aureus. Various bioactive compounds including polyphenols have been found in cocoa pods, which have essential properties such as antibacterial effects.

**Material and Methods:** This was an in vitro laboratory experimental study. Cocoa pod husk were extracted using ultrasonic method with ethanol 70% then formulated into gel with dosage of 20 mg/ml, 40 mg/ml, 80 mg/ml, 160 mg/ml, and 320 mg/ml. The antimicrobial activity was determined in the extracts using agar disc diffusion method, compared with chlorhexidine gel (positive control), and carbopol gel (negative control). Statistical analysing used kruskall wallis and Mann whitney test.

**Results:** Inhibition zone ranging from 6.62±0.51 to 15.06±0.63 mm was formed in group of cocoa pod husk ethanol extract gel at concentration 80 mg/ml, 160 mg/ml, and 320 mg/ml. It was indicated that there was antibacterial effects against Staphylococcus aureus. There were significant differences between sample groups (p<0.05).

**Conclusion:** Ethanol extract gel of cocoa pod husk (Theobroma cacao L.) can inhibit the growth of staphylococcus aureus at concentration 80 mg/ml, 160 mg/ml, and 320 mg/ml.

Keywords: Cocoa pod husk extract gel, Inhibition zone, Staphylococcus aureus

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Introduction

Staphylococcus aureus infection in the oral cavity causes various diseases like gingivitis, angular cheilitis, parotitis, staphylococcal mucositis, denture stomatitis, and abscesses. Abscess is a typical infection caused by S. aureus. Other oral infections associated with S. aureus include infection of jaw cysts and oral mucosal lesions. Staphylococcus aureus produces a number of virulence factors which aid in adherence to host cells, avoid host immune response, tissue invasion, cause sepsis and induce toxin-mediated syndromes. The process of S. aureus infection involves five stages, colonization, local infection, systemic spread and/or sepsis, metastatic infection and toxinois.

One of the antimicrobial agents used to treat cases of S. aureus infection is chlorhexidine. Chlorhexidine is a cationic bisbiguanide which is stable as a salt (chlorhexidine gluconate) used in concentrations ranging from 0.2% - 2%. Chlorhexidine is an antimicrobial agent that is active against viruses, fungi and bacteria. Currently chlorhexidine is routinely used by dentists to treat patients with periodontal care, orthodontia and maxillofacial surgery. Chlorhexidine is available in the form of mouthwash, soap, gel, spray, toothpaste and varnish with different strengths. Chlorhexidine has a broad-spectrum antibacterial agent which has strong effectiveness on S. aureus bacteria and is also effective as a treatment and prevention therapy for Methicillin Resistance Staphylococcus aureus (MRSA).

Prolonged use of chlorhexidine can cause side effects. The most common side effects of chlorhexidine use are discoloration of the teeth, restorations and tongue. Desquamative lesions of the oral mucosa have also been reported in a small number of individuals due to long-term chlorhexidine. Therefore, to minimize the side effects of synthetic antibiotics, an alternative antimicrobial therapy from natural ingredients.

The antibacterial properties of cocoa pod husk extract have been reported in several previous studies. Cocoa pod husk extract has an antibacterial effect in inhibiting streptococcus mutans. Other studies have also proven that proanthocyanidins extract of cocoa pod husk has the ability to inhibit S. mutans and P. gingivalis with minimum inhibitory concentration (MIC) of 16 mg/ml and 8 mg/ml. These results were also supported by other studies which reported that the extract of cocoa pod husk inhibited the growth of S. aureus, B. subtilis, and E. coli with a minimum inhibitory concentration of 8 g/ml, 16 g/ml, and 32 g/ml. Some of these studies were still limited to research on the identification of antibacterial activity and have not been further investigated into drug dosage forms. Based on this description, the researcher wanted to conduct research on the inhibition of cocoa pod husk extract.
in the form of a gel formulation against S. aureus bacteria. The results of these study were expected to be utilized and developed as a reference in making antibacterial gels for oral bacterial infections.

**Material and Methods**

This research was an in vitro laboratory experimental study with a posttest only control group design. It was approved by the Health Research Ethics Commission, Faculty of Dentistry, Jember University. The cocoa peel (Theobroma cacao L.) has been identified by the Jember State Polytechnic Plant Laboratory. The Materials used were erlenmeyer (Pyrex, Jepang), digital caliper (Inoki, Japan), micropipette (Eppendorf, Germany), microscope (Olympus, Japan), spectrophotometer (Milton Roy, Germany), ultrasonic homogenizer (Elmasonic), laminar flow cabinet (type HF-100, Korea), desicator (Kartell, Italia), incubator (WTC Binder, Jerman), vacuum rotary evaporator, autoclaf (Memmert, Germany), thermylone (Maxi Mix II, Dubuque, Lowa, USA), dry heat oven (Memmert Germany), Staphylococcus aureus strain ATCC 25923.

**Procedure of cocoa pod husk extraction and gel preparation**

The cocoa pod husk was cleaned and cut into small pieces and then oven for 2x24 hours at a temperature of 50°C then blended until it becomes a powder and sieved until a fine, homogeneous powder is obtained. A total of 150 grams of cacao pod were put in a beaker glass and added with 70% ethanol solvent as much as 600 ml and then homogenized with ultrasonic homogenizer for 10 minutes at a speed of 70 rpm. The mixture is separated from the residue using vacuum filtration and concentrated using a rotary vacuum evaporator then oven at 50°C for 2x24 hours. The solution that has been roasted is separated between the liquid part and the thick part and the final result is the crude extract of 100% cocoa peel. The extract was then formulated with Carbopol and Triethanolamine (TEA) to make gel preparations consisting of doses of 20 mg/ml, 40 mg/ml, 80 mg/ml, 160 mg/ml, and 320 mg/ml. The samples used consisted of 5 treatment groups and 2 control groups. The treatment group using ethanol extract gel of cocoa pod husk 20 mg/ml, 40 mg/ml, 80 mg/ml, 160 mg/ml, and 320 mg/ml. Positive control group was chlorhexidine gel and negative control group was gel base Carbopol and Triethanolamine (TEA).

**Inhibition zone against S. aureus**

The collection of bacteria strain of S. aureus based on the American Type Culture Collection, ATCC 25923. The bacterial suspension was made by taking 1 ose of the bacteria culture and then adding 2 ml of Brain Heart Infusion Broth (BHI-B) media in a test tube, then the test tube mouth was passed to a fire bunsen then homogenized by centrifuge. The tubes were then incubated for 24 hours at 37°C. The incubated bacterial suspension was taken using a micropipette and put into a test tube containing 3 ml of BHI-B solution and vibrated with thermylone then measured the absorbance of 0.3 McFarland using a spectrophotometer. If the suspension is too cloudy, it can be added with BHI-B. Inhibition zone of extract gel of the cocoa peel against the growth of S. aureus was carried out using the disk diffusion. The bacterial suspension was inoculated on MHA media. 4 Petridishes were incubated at 37°C for 24 hours, and the formed inhibition zone was measured using a caliper. The inhibition zone that is formed is a clear zone where there is no bacterial growth.

**Statistical Analysis**

The results of the inhibition zone diameter were then analyzed statistically using the SPSS 24.0. The study was tested for normality using the Shapiro Wilk test and the homogeneity test using the Levene test. Furthermore, a non-parametric test was carried out using the Mann Whitney and Kruskall-Wallis tests showed the significant differences between sample groups (p<0.05).

**Results**

Measurement of inhibition of cocoa pod husk ethanol extract gel against the growth of S. aureus resulted in an inhibition zone formed around the paper disk in several sample groups. In positive control group, the cocoa pod husk extract gel group of 80 mg/ml, 160 mg/ml, and 320 mg/ml after incubation for 24 hours. The results of the large diameter of the inhibition zone of each treatment group are shown in table 1 and figure 1.

The results of this study showed that the response of a substance to bacteria is classified into 4 levels of inhibition zones. This level is in the weak category if the diameter of the inhibition zone formed is less than 5 mm, the moderate category if the inhibition zone ranges from 5-10 mm, the strong category is 10-20 mm, and very strong inhibition zone formed is more than 20 mm. The ethanol extract gel of cocoa pod husk 80 mg/ml was included in the moderate category based on the average inhibition zone formed, which is 6.62 mm. The ethanol extract gel of cocoa
Table 1 Diameter of inhibition zone for ethanol extract gel of cocoa pod husk (Theobroma cacao L.) on Staphylococcus aureus

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Diameter of inhibition (mm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>4</td>
<td>12.99 ± 1.36</td>
</tr>
<tr>
<td>Negative control</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CPE 1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CPE 2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CPE 3</td>
<td>4</td>
<td>6.62 ± 4.51</td>
</tr>
<tr>
<td>CPE 4</td>
<td>4</td>
<td>10.85 ± 1.18</td>
</tr>
<tr>
<td>CPE 5</td>
<td>4</td>
<td>15.06 ± 0.63</td>
</tr>
</tbody>
</table>

Note:
N: the number of samples
The positive control = chlorhexidine gel
The negative control = gel base
CPE 1 = Cacao peel extract 20 mg/ml
CPE 2 = Cacao peel extract 40 mg/ml
CPE 3 = Cacao peel extract 80 mg/ml
CPE 4 = Cacao peel extract 160 mg/ml
CPE 5 = Cacao peel extract 320 mg/ml

Discussion
The zone of inhibition that forms around the disc is caused by the presence of active substances contained in the material being tested. The measurement of the inhibition zone aims to determine the amount of active substance release observed based on the diameter of the inhibition zone formed. Ethanol was a universal solvent so that both polar and nonpolar compounds could be extracted optimally, alongside ethanol being an easy-to-obtain and harmless solvent such as methanol. Ethanol had a low toxicity level and a versatile solvent. The extraction of medicinal plant material with ethanol solvent into a liquid extract or dried extract was mostly done for the purpose of standardization of herbal medicine.

The selection of chlorhexidine gel which is an antimicrobial agent as a positive control for the comparison of the inhibition of ethanol extract gel of cocoa pod husk in this study was based on several studies which showed that S. aureus had a tendency to be resistant to antibiotics. Research conducted by Abdallah et al. showed that S.aureus resistance was not found due to prolonged use of chlorhexidine, so based on some of these results, a positive control was used for chlorhexidine in this study to minimize the absence of antibacterial activity due to S.aureus resistance to antibiotics.

The active compounds in the cocoa pod husk which is thought to play a role in inhibiting the growth of S. aureus includes alkaloids and flavonoids. Phytochemical examination of the ethanol extract of cocoa pod husk showed positive results detected by the presence of alkaloids and flavonoid compounds. Alkaloid compounds are organic compounds that have nitrogen atoms and are alkaline and can cause protein coagulation of bacterial cells so that they interfere with the peptidoglycan constituent components of bacterial cells which cause the cell wall layer to not form completely and the bacterial cell to experience death.

Tannins are also one of the active compounds contained in the cocoa pod husk which have antibacterial effect. Tannins work by targeting the polypeptides of the bacterial cell wall so that the formation of the cell wall is imperfect which can lead to bacterial lysis. Tannins have been shown to inhibit the growth of Gram-positive and Gram-negative bacteria. Tannins can also inhibit the attachment of bacteria to the cell surface, causing cell death. The absorption of sugars and amino acids can also be inhibited by tannins so that bacteria do not have an energy source.

Triterpenoids are also known to be active compounds contained in cocoa peel that have antibacterial properties. Triterpenoids are one of the terpenoid class compounds that work by reacting with porin (transmembrane protein) on the outer membrane of the bacterial cell wall. Triterpenoids form strong polymer bonds that dam-
The antibacterial activity of cocoa pod husk extract is thought to have high antioxidant activity. The antioxidant activity of a plant is generally caused by the presence of phenolic compounds, both polyphenols and simple phenols. Phenolic compounds can be in the form of flavonoids and tannins which are polyphenols.

Saponins is an active compound in cocoa peel act as antibacterial agents that work by hydrolyzing the bacterial cell walls. The metabolism of bacterial cells will then be disrupted and the process of ATP formation for cell growth will be inhibited. If this process continues, it will cause cell death. Saponins destroying bacterial proteins, will damage the integrity of the cell membrane, thereby causing bacterial cell death.

The results of this study also indicated that the ethanol extract gel of cocoa pod husk 20 mg/ml and 40 mg/ml have the same antibacterial activity as the negative control. The ethanol extract gel of cocoa pod husk 80 mg/ml has less antibacterial effect than 160 mg/ml and 320 mg/ml. The difference in antibacterial activity is due to the concentration of the active compound contained in the ethanol extract gel of the cocoa pod husk varies according to the concentration.

The antibacterial activity of the cocoa pod husk extract is also influenced by the structure of the gram-positive bacteria cell wall, which has a tightly arranged peptide chain between the glycan chains one another, causing the structure of the cell walls to be more difficult to penetrate by compounds from outside. The ethanol extract gel of cocoa pod husk at 20 mg/ml and 40 mg/ml contained a small amount of active compounds, so it was thought that they were not sufficient to inhibit the growth of S. aureus bacteria. The concentration of extract, type of antimicrobial materials and solvents also determined the ability of bacterial growth inhibition. The limitations of a study were need the minimum inhibitory concentration (MIC) test to demonstrate the lowest level of antimicrobial agent that greatly inhibits growth, and minimum bactericidal concentration (MBC) to demonstrate the lowest level of antimicrobial agent resulting in microbial death.

Conclusion
Ethanol extract gel of cocoa pod husk has antibacterial activity against Staphylococcus aureus at doses of 80 mg/ml, 160 mg/ml, and 320 mg/ml. For the future, research on the antibacterial effects of cocoa pod husk still needs further studies on other gram-negative and gram-positive bacteria to strengthen the scientific evidence.

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Conflict of Interest
The authors report no conflict of interest.

References
5. Panesa MR. Effectiveness of inhibitory power of cherry leaf ethanol extract compared to 0.2% chlorhexidine gluconate against staphylococcus aureus (in vitro research on heat cured acrylic resin plate). Dentin 2018;2: 79-84. (In Indonesia)
11. Pangemanan SP, Edy HJ, Rumondor EM. Test the effectiveness of the formulation of goroho banana peel extract cream (Musa acuminafe L) against staphylococcus aureus bacteria. Pharmaco 2020;9: 443-450. (In Indonesia)

