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Antibacterial Activity of Ethanol Extract of Cocoa Beans (*Theobroma cacao* L.) on *Porphyromonas gingivalis* and *Streptococcous mutans*

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Abstract

The aim of this study was to determined the antibacterial activity cocoa beans (*Theobroma cacao* L.) ethanol extract on *Porphyromonas gingivalis* and *Streptococcous mutans*. Various bioactive compounds including polyphenols have been found in cocoa beans, which have essential properties such as antibacterial effects. This was an in vitro laboratory experimental study. Cocoa beans were extracted using ultrasonic method with ethanol 70%, then divided into groups of cocoa bean ethanol extract (CBE) with a concentration of 100%, 75%, 50%, 25%, compared with positive and negative control groups. The antimicrobial activity was determined in the extracts using agar disc diffusion method, and statistical analysing used Anova. The inhibition zone of strong category on *P.gingivalis* was formed in CBE group at concentration 50%-100%, while on S. mutans was formed in CBE group at concentration 25%-100%. The average diameter of inhibition zone against *S. mutans* was larger than *P. gingivalis*. There were significant differences between sample groups ($p<0.05$). It was indicated that there was antibacterial effects against *P. gingivalis* and *S. mutans*.

Keywords: Cocoa Beans; Inhibition Zone; *Porphyromonas gingivalis*; *Streptococcous mutans*

Introduction

Malocclusion is a dental and oral health problem that occurs due to an abnormality in the position or relationship of the jaw and teeth, either between teeth in one arch or with teeth in the opposing arch. Orthodontic treatment aimed at correcting malocclusion is closely related to periodontal complications, such as gingivitis, gingival recession or hypertrophy, periodontitis, and caries [1]. Gingivitis is an inflammatory condition of the gingival tissue, most often caused by bacterial infection, which is also a form of periodontal disease with inflammation of the gingival tissue characterized by swelling, redness, pain, and bleeding caused by the presence of microbial plaque that settles in the

gingival sulcus [2].

Microbial plaque includes gram-negative bacteria, such as Porphyromonas gingivalis which plays a role in the development of gingivitis and periodontitis. In addition, Streptococcous mutans is a gram-positive bacteria that is often found in the oral cavity and has the ability to form plaque and caries [3]. Streptococcus mutans is also a cariogenic bacteria that contributes significantly to the pathogenesis of cavities. Both of these bacteria have a important role in the formation of plaque and biofilm, which are major factors in the development of oral health problems [4].

Cocoa bean extract has a higher content of nutrients and bioactive substances than the skin and fruit. The derivative of the largest polyphenol compound is flavonoids with a percentage of 12-18% consisting of three main groups, namely proanthocyanins 58%, catechins 37% and anthocyanins 4%, which act as natural antioxidants in oral health, in addition to their anti-inflammatory and antibacterial properties [5]. Polyphenols can quickly change the characteristics of the dentin surface, especially through interactions with collagen and enamel, to provide superior adhesive ability and antibacterial activity against various microbes in the oral cavity [6]. The aim of this study was to analyze the antibacterial potential of bioactive compounds from cocoa bean (*Theobroma cacao* L.) extract on *Porphyromonas gingivalis* and *Streptococcus mutans*.

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 beans (*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 (tipe HF-100, Korea), desicator (Kartell, Italia), incubator (WTC Binder, Jerman), vacuum rotary evaporator (Memmert, Germany), thermolyne (Maxi Mix II, Dubuque, Lowa, USA), dry heat oven (Memmert Germany), *Porphyromonas gingivalis* strain ATCC 33277, *Streptococcus mutans* strain ATCC 25175.

Procedure of Cocoa Beans Extraction

The cocoa pod husk was cleaned and cut into small pieces and then oven for 2x24 hours at a temperature of 50ºC then blended until it becomes a powder and sieved until a fine, homogeneous powder is obtained. A total of 150 grams of cacao beans 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 50ºC 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 beans [7].

Incubation of Bacterial Suspension

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 37^0C. 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 thermolyne then measured the absorbance of 0.3 McFarland using a spectrophotometer. In the suspension can be added with BHI-B. The number of observations consisted of 4 repetitions of each bacteria, which were divided into groups of cocoa bean ethanol extract with a concentration of 100%, 75%, 50%, and 25%, as well as positive control (Chlorhexidine) and negative control (Aquadest).

Inhibition Zone against *P. gingivalis* and *S. mutans*

These methods rely on the diffusion of anti-microbial agents from paper discs, inhibiting the growth of the test microorganism inoculated on the agar surface measuring the resulting zone of inhibition [8]. Inhibition zone of extract gel of the cocoa beans against the growth of S. mutans was carried out using the disk diffusion. The bacterial suspension was inoculated on MHA media. Four 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 Chlorhexidine zone where there is no bacterial growth [9].

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 beans ethanol extract (CBE) against the growth of *P. gingivalis* resulted in an

inhibition zone formed around the paper disk in several sample groups. In positive control group, CBE group of consentration 0f 100%, 75%, 50% and 25% after incubation for 48 hours. The results of the large diameter of the inhibition zone of each treatment group are shown in Table 1 and Figure 1.

Groups	N	Diameter of inhibition p (mm ± SD)
Positive control	4	20.95 ± 1.18 0.00*
Negative control	4	0
CBE 25%	4	9.55 ± 1.67
CBE 50%	4	12.60 ± 1.36
CBE 75%	4	15.20 ± 0.63
CBE 100%	4	17.00 ± 1.88

N: the number of samples (repetitions), positive control: chlorhexidine gel, negative control group: aquadest, CBE: cocoa bean ethanol extract. *Significant p<0.05.

Table 1: Diameter of inhibition zone of of cocoa beans ethanol extract (CBE) on *P. gingivalis.*

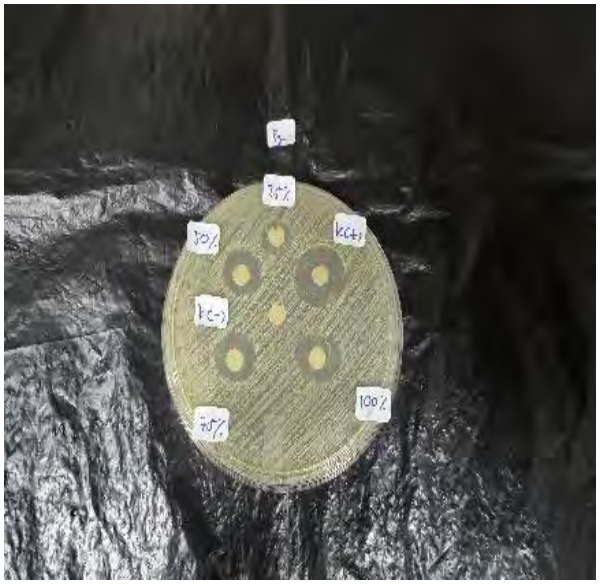

Figure 1: Inhibition zone of CBE on the growth of *P. gingivalis.*

Measurement of inhibition of cocoa beans ethanol extract (CBE) against the growth of *S. mutans* resulted in an inhibition zone formed around the paper disk in several sample groups. In positive control group, CBE group of consentration 0f 100%, 75%, 50% and 25% after incubation for 48 hours. The results of the large diameter of the inhibition zone of each treatment group are shown in Table 2 and Figure 2.

Groups	N	Diameter of inhibition p (mm ± SD)
Positive control	4	21.40 ± 1,23 0.00*
Negative control	4	0
CBE 25%	4	10.95 ± 0.77
CBE 50%	4	13.60 ± 2.33
CBE 75%	4	15.95 ± 0.83
CBE 100%	4	18.33 ± 1.67

N: the number of samples (repetitions), positive control: chlorhexidine gel, negative control
group: aquadest, CBE: cocoa bean ethanol extract. *significant p<0.05.

Table 2: Diameter of inhibition zone of of cocoa beans ethanol extract (CBE) on *S. mutans.*

Figure 2: Inhibition zone of CBE on the growth of *S. mutans.*

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 [10]. The CBE25% was included in the moderate category based on the average inhibition zone formed. The CBE 50% and 75% was included in the strong category, with an average diameter of the inhibition zone formed, 11-18 mm. Chlorhexidine as positive control was a very strong category. Base on the results can be assumed that CBE has antibacterial activity in inhibiting the growth of *P. gingivalis and S. mutans.*

Discussion

Flavonoids and phenolic acids are significant groups of polyphenol compounds because they can directly

affect bacterial growth and reduce pathogen activity. Antioxidants with antibacterial properties work through three basic mechanisms, namely increasing outer membrane permeability, cytoplasmic leakage, and inhibiting nucleic acid production. The antibacterial effect of polyphenols can be explained by their ability to bind iron, which is essential for the survival of most bacteria. Polyphenols damage bacterial cell walls, increase cytoplasmic membrane permeability, and release lipopolysaccharides [7].

Cocoa bean extract contains various active compounds, including flavonoids, polyphenols, tannins, and alkaloids that play an important role in antibacterial activity. Flavonoids, which are one of the main compounds in cocoa beans, have the ability to interact with bacterial cell membranes. Flavonoids can also affect the structure of bacterial cell walls, increase cell membrane permeability, and ultimately lead to bacterial cell damage [11]. Polyphenols in cocoa beans also have strong antibacterial effects. Polyphenols work by damaging the bacterial cell membrane, as well as disrupting the metabolic processes and cell division of bacteria [12].

Alkaloid compounds of *T. cacao*, such as theobromine, which are known to have antibacterial effects against several types of bacteria, including *S. mutans*. Theobromine can affect the bacterial metabolic system by inhibiting the activity of certain enzymes needed by bacteria to survive [13]. This alkaloid also has the potential to inhibit biofilm formation, which is one way *S. mutans* survives in the oral cavity.

In this study, the antibacterial activity of cocoa bean extract was shown to be effective against *S. mutans,* which plays an important role in the formation of dental plaque and gum disease and *P. gingivalis,* which is associated with periodontitis. The cocoa bean extract showed an inhibition zone which proved that the cocoa bean extract had the ability to inhibit the growth of *P. gingivalis and S. mutans* bacteria, although smaller when compared to Chloroxidine. However, although cocoa bean extract with a higher concentration provided a larger inhibition zone, at very high concentrations, there is a possibility of toxicity or irritation to human body cells. Therefore, further research is needed to determine the right dosage and potential side effects of using cocoa bean extract as an antibacterial agent. Although natural extracts have high antibacterial potential, their use must be considered so as not to cause unwanted side effects in humans [14].

S. mutans is a bacteria commonly found in the oral cavity and is the main cause of tooth decay (caries) through the production of acid from carbohydrate fermentation. While *P. gingivalis* is more associated with periodontal disease [15]. This study shows that cocoa bean extract has stronger antibacterial activity against *S.mutans*, possibly due to differences in the pathogenetic mechanisms of the two bacteria. *S. mutans* is more sensitive to cocoa extract due to the presence of antibacterial compounds in cocoa bean extract that can directly interfere with the metabolism of this bacteria. This is due to the different mechanisms of action of the bacterial growth inhibitory power. Cocoa bean extracts are also able to inhibit the growth of *P. gingivalis*, which produces many virulence factors that contribute to its pathogenicity, including cysteine proteinases (also called gingipains), which are responsible for proteolytic activity [16].

Based on the results of this study, cocoa bean extract can be a potential alternative to prevent or treat dental caries caused by *S. mutans.* Cocoa bean extract contains bioactive compounds that can help inhibit the growth of *S. mutans* and prevent the formation of dental plaque, which is a major factor in the development of caries. The use of cocoa bean extract in dental care products, such as natural toothpaste or mouthwash, can be an interesting option. Such products are increasingly in demand because they are considered safer and more environmentally friendly compared to products made from synthetic chemicals. In addition, the use of natural ingredients such as cocoa bean extract can reduce the potential for bacterial resistance to antibiotics, which is increasingly becoming a global problem [17]. However, although cocoa bean extract shows good antibacterial potential, further research is needed to test the right dosage and ensure its effectiveness and safety in humans.

Conclusion

Ethanol extract of cocoa beans (*Theobroma cacao* L.) has antibacterial activity against Porphyromonas gingivalis and Streptococcus mutans, and showed significant differences ($p < 0.05$) between treatment and control. Antibacterial activity test showed that the higher the concentration of the extract, the larger the inhibition zone formed, indicating an increase in antimicrobial effectiveness.

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