DOI: http://dx.doi.org/10.33846/hn50805
http://heanoti.com/index.php/hn

RESEARCH ARTICLE
URL of this article: http://heanoti.com/index.php/hn/article/view/hn50805

Antibacterial Activity of Fermented and Non-Fermented Edel Cacao Bean Extract (*Theobroma Cacao L.*) Against *Porphyromonas gingivalis*

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ABSTRACT

Porphyromonas gingivalis is considered the primary etiologic factor in periodontitis. The administration of antibiotics has so many adverse effects that alternative ingredients are needed with minimal side effects and as an agromedical-based innovation from herbal ingredients abundant in Jember district, namely extracts from cocoa beans. This study aimed to determine the antibacterial activity of fermented and unfermented cocoa bean extract (*Theobroma cacao L.*) with various concentrations against P. gingivalis. This research was conducted using fermented cocoa bean extract 1%, 2%, 3%, unfermented cocoa bean extract 1%,2%,3%, chlorhexidine as a positive control, aquadest as a negative control. Extracts were made using the Ultrasound-Assisted Extraction method, then tested for antibacterial using the disk diffusion method on Muller Hinton Agar media, four replications, 24 hours incubation, and measuring the inhibition zone with a caliper. The minimum inhibitory concentration of fermented cocoa bean extract was 3%, the size of the inhibition zone was 12.57 mm, the unfermented cocoa bean extract was 2%, the size of the inhibition zone was 11.67 mm. Test the difference using Kruskal walis, significance value 0.001. Antibacterial activity of cocoa bean extract (Theobroma cacao L.) unfermented Edel variety had more potent antibacterial activity against P. gingivalis than fermented one. The results of the Mann-Whitney test between samples showed that there were almost significant differences between the researched samples.

Keywords: antibacterial; *Porphyromonas gingivalis*; cocoa beans

INTRODUCTION

The main problem of teeth and mouth that mostly suffers in Indonesia is dental caries, then in the second rank is periodontal tissue disease, namely gingivitis and periodontitis[(1)]. Periodontitis is an inflammatory disease that affects the periodontium[(2)]. According to the World Health Organization, periodontal disease affects 10-15% of the adult population worldwide[(3)]. According to Riskesdas 2018, in the age range of 35-44 years, the prevalence of periodontitis was found to be very large, reaching 77%, and in all cases in Indonesia, it reached 74.1%. Periodontitis is a problem that needs attention[(1)]. Periodontitis is an inflammatory disease that affects the periodontium. Periodontitis involves progressive loss of the alveolar bone around the teeth. If left untreated, it can lead to loosening and bone loss at a slow rate, characterized by pocket formation and gingival recession, leading to tooth loss. Periodontitis is caused by microorganisms that attach to and grow on the tooth surface, together with an overly aggressive immune response to these microorganisms[(2)]. P. gingivalis is a gram-negative oral anaerobic bacterium and is considered to be the primary etiologic factor in periodontitis disease with a large number of virulence factors and extracellular proteases by exotoxin or endotoxin[(4, 5, 6)].

Administration of this systemic antibiotic drug must reach a concentration or dose sufficient to have a therapeutic effect to kill or inhibit the growth of the target microorganism. The drugs that are often used are metronidazole, ciprofloxacin, amoxicillin, and other combinations of drugs. When administering systemic antibiotics, an aspect to consider is that the medication must reach a location where the organism present maintains its local concentration at a sufficient level for an adequate time and causes minimal or no side effects. Considering these factors, the use of local antibiotics is the right choice. One of the local antibiotics that are effective and can be used is chlorhexidine. However, the use of antibacterials poses a risk of adverse reactions to the body other than the oral cavity, which can cause nausea, vomiting, headache, urticaria, gastrointestinal disturbances, stomach discomfort, allergic reactions, bacterial resistance in patients, and extrinsic discoloration of the teeth[(2, 7)].

In this paper, considering the benefits and side effects of long-term adverse for the administration of antibacterial agents, an alternative material with minimal side effects is needed and is an agromedical-based innovation from herbal ingredients that are very abundant in the Jember district, namely extracts from cocoa beans. Cocoa bean extract contains active compounds, namely flavonoids, which are believed to be strong antibacterial agents[8]. Flavonoids are now considered indispensable components in various nutraceutical, pharmaceutical, pharmaceutical, and cosmetic applications. This is due to its antioxidant, anti-inflammatory, antimutagenic, antibacterial, and anticarcinogenic properties, coupled with its capacity to modulate the function of major cellular enzymes[9]. The amount of flavonoid polyphenols varies due to the type of variety and country of origin of the cocoa beans and depends on the cocoa beans' production process [10]. These cocoa production processes start with cacao planting, storage, fermentation, drying, and packaging of cacao beans, then continue with chocolate making. The amount and composition of amino acids change during the fermentation process and reduce sugars such as glucose and fructose, polyphenols, and pH profile. The presence of yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) during the fermentation process positively affects the polyphenol concentration and the development of flavor pioneers. At the same time, spores and aerobic fungi have a negative effect. This fermentation influences the bioactive content of cocoa, microbiological activity, and enzymes[10].

Based on the description above, it is desirable to know the difference in antibacterial activity of fermented and unfermented cocoa bean extract (Theobroma cacao L.) against P. gingivalis bacteria as a preliminary test through experimental research conducted in vitro using the diffusion method.

METHODS

This researched was experimental laboratory researched with a studied design that was post-test only controlled group design. Cocoa beans (*Theobroma cacao L.*) fermented Edel variety was obtained from PTPN Kedaton with a spontaneous fermentation process assisted by microorganisms in the fermentation process. The cocoa fermentation process generally took place naturally for five days of fermentation with the helped of yeast, lactic acid bacteria, and acetic acid bacteria. Cocoa beans (*Theobroma cacao L.*) of the unfermented Edel variety were beans obtained from PTPN Kedaton without a spontaneous fermentation process

Cocoa beans identified were 2 kg each extracted used the Ultrasound-Assisted Extraction (UAE) method used 96% ethanol as solvent to maintained and produced higher yields quickly and did not damage the compounds in the extract due to heating. This method used the helped of ultrasonic waves, namely the amplitude that could be caused cavitation for better penetration of the solution on the walls and cell membranes of cocoa beans used an ultrasonic cleaner with an operating time of 2x30 minutes[11][12]. Extracts were made at the Pharmacy Laboratory of the University of Jember to produced 6. 9 grams of fermented cocoa bean extract and 8. 61 grams of unfermented cocoa bean extract.

The extract results were divided into six treatment groups. The treatment group consisted of fermented and unfermented cocoa bean extract with 1%, 2%, and 3% concentrations, respectively. The controlled group was a positive controlled used chlorhexidine, and the opposing group used aquadest

All groups were then tested on P. gingivalis bacteria obtained from the Airlangga University Researched Center with the product name ATCC 33277 PK/5. After being tested for gram staining, it was proven to have been Gram-negative bacteria. These bacteria were cultured on BHIB media and then tested for antibacterial on Muller Hinton Agar media for 24 hours following the bacterial growth phase

Testing of antibacterial activity used the disk diffusion method for 1x24 hours on Muller Hinton Agar's Petri dished media was carried out by looking at the cleared area around the 6mm paper disc whose diameter parameter was calculated in millimeters from the outermost point of the circle past the centerline of the disc paper used a ruler or caliper.

The data from each group were analyzed used the Kruskal Wallis non-parametric statistical test. The data was not homogeneous used the Levene test and normally distributed used the Shapiro-Wilk test. Furthermore, the Mann-Whitney test was carried out to saw significant differences between the researched samples

RESULTS

Based on the researched results carried out, it could be referred to in figure 1. It could be seen that there was an inhibition zone in the fermented cocoa bean extract group referred to in the Petri dished. Figure 1 (a) was divided into five pieces of sample groups in each Petri dished, namely group 1 was fermented cocoa bean extract with a concentration of 1%, group 2 was fermented cocoa bean extract with 2% concentration, group 3 was fermented cocoa bean extract 3%, a positive controlled group was used chlorhexidine (+), and the negative controlled group was used aquadest (-).

The unfermented cocoa bean extract was referred to in the Petri dished of figure 1 (b), which was divided into 5 sample groups in each Petri dished, namely group 1 was an extract of unfermented cocoa beans with a concentration of 1%, group 2 was an extract of unfermented cocoa beans with a concentration of 2%, group 3 was 3% unfermented cocoa bean extract, the positive controlled group was used chlorhexidine (+), and the negative controlled group was used aquadest (-)

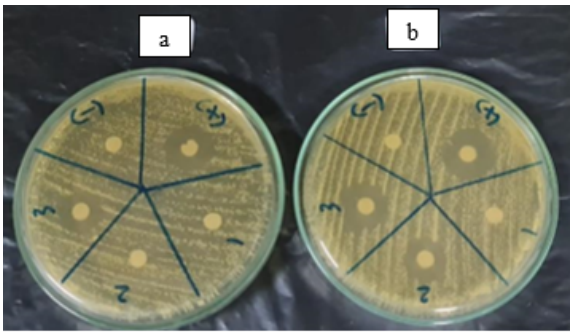

Figure 1. The image of the transparent area around the paper disc on Muller Hinton Agar media for P. gingivalis bacteria has been treated with cocoa bean extract as the test material. Bean extract group Fermented cocoa bean extract group (a) and unfermented cocoa (b)

Based on these observations, the inhibition zone was measured used a ruler or caliper with millimeter units. The results of the calculation of the average inhibition zone for unfermented cocoa bean extract and fermented cocoa bean extract could be referred to in Table 1.

Table 1. Data from the measurement of the inhibition zone of fermented and unfermented cocoa bean extract

Group	n	Mean	$\bar{x}$±SD (mm)
Negative Control (-)	8	0	0
Positive Control (+)	8	18.06	18.06±0.45
1% unfermented cocoa bean extract	4	0	0
2% unfermented cocoa bean extract	4	11.67	11.67±0.24
3% unfermented cocoa bean extract	4	13.43	13.43±0.17
1% fermented cocoa bean extract	4	0	0
2% fermented cocoa bean extract	4	0	0
3% fermented cocoa bean extract	4	12.57	12.57±0.14

Explanation: n: sample size; $\bar{x}$: average diameter of inhibition zone; SD: Standard deviation of inhibition zone diameter

Table 1 shows the results of the average inhibition zone measurement of each studied sample with four repetitions. It could be seen that there were differences in the results of the size of the inhibition zone of fermented and unfermented cocoa bean extract against the Muller Hinton agar media of P. gingivalis bacteria. Unfermented cocoa bean extract could affect bacterial colonies and form an inhibition zone at a concentration of 2%, while fermented cocoa bean extract was at a concentration of 3%. The positive controlled, namely chlorhexidine, had the most extensive diameter inhibition with an average valued of 18,06 mm. The 3% unfermented cocoa bean extract had an average inhibition zone diameter of 13,43 mm. The unfermented cocoa bean extract with a concentration of 2% had an average inhibition zone diameter of 11,67 mm. Fermented cocoa bean extract with a concentration of 3% had an average of 12,57 mm.

The results of the average inhibition zone showed that the unfermented cocoa bean extract had a larger diameter than the fermented cocoa bean extract. Unfermented cocoa bean extract concentration as an antibacterial against p. gingivalis had a more potent inhibitory concentration than fermented cocoa bean extract. At a concentration of 2%, unfermented bean extract had been able to act as an antibacterial. In comparison, the fermented cocoa bean extract could only serve as an antibacterial at a concentration of 3%

The overall data from each group's results were analyzed using the Kruskal Wallis non-parametric statistical test. The data were normally distributed, tested by Shapiro Wilk, and the Levene test tested not homogeneous. In the Kruskal Wallis test, the significance valued was less than 0. 05, i.e, 0. 001 each, meaning that there was a significant/significant difference in the inhibition zone's value between the concentrations of the studied samples that affected the antibacterial activity. Furthermore, the Mann-Whitney test was carried out to saw significant differences between the researched samples

The results of the Mann-Whitney test between samples showed that there was almost significant differences between the researched samples, except for the fermented cocoa bean extract at concentrations of 1% and 2%, 1% concentration and negative controlled, 2% concentration and negative controlled, and unfermented cocoa bean extract 1% concentration and negative controlled. This happened because the results of the inhibitory zone of the cocoa bean extract were the same as could be referred to in Table 1. It was stated that no inhibition zone was found so that at these concentrations, both did not affect the antibacterial activity. So the difference was significant, with values below 0,005 in the other samples.

DISCUSSION

This study aimed to determine the antibacterial activity of fermented and unfermented cocoa bean extract (*Theobroma cacao L.*) with various concentrations against *P. gingivalis*. The production of fermented and unfermented Edel cocoa bean extract was carried out using the Ultrasound-Assisted Extraction method. This method uses ultrasonic waves using 96% ethanol as a solvent. Ultrasonic waves that are amplitude can cause cavitation for better penetration of the solution on the walls and cell membranes of the extracted material[11].

This Ultrasound-Assisted Extraction method was chosen to maintain and produce higher yields quickly and not damage the compounds in the extract due to heating[11, 12]. Secondary metabolite compounds such as flavonoids and phenolics are easily damaged at high-temperature heating. The choice of this method is because flavonoids are phenolic compounds that have a conjugated aromatic system that is easily damaged at high temperatures[13]. A number of research results show that the application of ultrasonic intensity (amplitude) techniques can be applied to the process of extracting phytochemical compounds, such as alkaloids, flavonoids, polysaccharides, proteins, and essential oils from various plant parts and plant seeds[11].

As a solvent, ethanol can cause polyphenols to be easily soluble in solvents. This happens because of the polar nature of the polyphenols in cocoa beans which can be stabilized with a mixture of acidic solutions[14]. Ethanol is proven to be better and is the right choice for the extraction of flavonoid content compared to aquadest, acetone, and methanol solvents[15]. Cocoa bean extract (*Theobroma cacao L.*) fermented and unfermented Edel varieties were made into several concentrations, namely 1%, 2%, and 3%. The extract was then tested against *P. gingivalis* bacteria to see its antibacterial activity using the media's disk diffusion method.

The principle of this method is that a paper disc with a certain diameter is moistened with a solution of cocoa bean extract, then placed on an agar plate that has previously solidified, namely on a surface that has grown bacterial cultures[16]. This paper disc method has the advantage that more tests can be carried out in one activity. It is easier to measure the area of the inhibition zone formed because the isolates can move from the top surface of the nutrient to the bottom and require minimal energy. The drawback is that there are many factors that affect the medium, the type of media, the inoculum, and the rate of diffusion of the antibacterial agent, so it is not known for certain whether the inhibition is bactericidal or bacteriostatic[17]. The media used is Muller Hinton Agar. According to the American Society for Microbiology, it is the best medium and is in accordance with the Clinical and Laboratory Standards Institute (CLSI) because it is a non-selective and undifferentiated medium (all bacteria can grow), low in sulfonamides, trimethoprim, and tetracycline, and contains starch that can absorb toxins released by bacteria, so it does not interfere with the cocoa bean extract. This antibacterial test will form a clear area around the paper disc[18, 19].

Based on research that has been done, the inhibition zones formed are seen in fermented cocoa bean extract with a concentration of 3%, unfermented cocoa bean extract with a concentration of 2%, unfermented cocoa bean extract with 3%, and positive control, namely chlorhexidine. The measurement of the diameter of the inhibition zone according to the criteria of Davis and Stout (1971) can be classified into several categories, and if when calculating the inhibition zone for the antibacterial test and the clear area is less than 5 mm in size, the inhibitory activity can be declared weak. If the result of the inhibition zone measurement is 5-10 mm, it is considered moderate. If the inhibition zone measurement results get a clear zone measuring 10-20 mm, it can be declared strong. If the clear zone is 20 mm or more, it is said to be very strong[20].

The difference in the diameter of the inhibition zone formed can be seen in a positive control, namely chlorhexidine with an average inhibition zone diameter of 18.06 mm, fermented cocoa bean extract concentration of 3% of 12.57 mm, 2% unfermented cocoa bean extract of 11 .67 mm, and 3% unfermented cocoa bean extract of 13.43 mm. The results of the research conducted on the chlorhexidine group, 3% fermented cocoa bean extract, 2% unfermented cocoa bean extract, and 3% unfermented cocoa bean extract, proved to have antibacterial activity with strong criteria based on the Davis and Stout criteria classification. Based on these results, the strongest inhibition zones were chlorhexidine, unfermented cocoa bean extract, and fermented cocoa bean extract. The difference in the strength of antibacterial activity by chlorhexidine with cocoa bean extract is because chlorhexidine has been shown to inhibit bacteria and is known to have broad-spectrum antibacterial activity against the growth of Gram-positive and Gram-negative bacteria[14].

The mechanism of chlorhexidine is that the cationic molecule chlorhexidine binds to negatively charged phosphate and carboxyl groups on the surface of bacterial cells facilitated by electrostatic forces. This process alters the integrity of the bacterial cell membrane, and the chlorhexidine molecules are attracted to the inner cell membrane. Furthermore, chlorhexidine binds to phospholipids in the inner membrane, causing increased permeability of the inner membrane and leakage of low molecular weight components such as potassium ions[2].

Based on the research results that have been carried out, it is proven that unfermented cocoa bean extract has antibacterial activity not much different from chlorhexidine, which is a local antibiotic that is often used in periodontitis therapy. Therefore, the unfermented Edel variety cocoa bean extract can be used as a natural antibacterial source from herbal ingredients and as an agromedical drug innovation. Cocoa bean extract is not fermented and fermented at a specific concentration is proven to cause the formation of an inhibitory zone. This is because the content of active compounds in the extract is one of the flavonoids. In fact, many studies have been conducted using cocoa bean extract against other bacteria, including by Hafidhah and Hakim (2017) against Enterococcus faecalis bacteria, by Kumalasari et al. in 2015 against the bacterium Pseudomonas aeruginosa, by Ariska (2015) against the bacterium Propionibacterium acnes, by Diyantika et al. in 2014 against *Staphylococcus aureus*[14, 21, 22, 23].

Flavonoids can be useful as antioxidants, anti-inflammatory, antimutagenic, antibacterial, and anticarcinogenic properties coupled with their capacity to modulate the function of major cellular enzymes[8, 9]. As an antibacterial,

flavonoids can inhibit other attachments and the growth of microorganisms[24]. Other compounds that can inhibit and have antibacterial properties of cocoa bean extract are catechins, procyanidins, and anthocyanins. Catechins are bactericidal by denaturing bacterial proteins. Procyanidins can inactivate or destroy genetic material in bacteria, while anthocyanins function as antibacterials[14]. However, the 1% and 2% unfermented cocoa bean extract and 1% and 2% fermented cocoa bean extract did not have antibacterial activity, likewise, in the negative control group with aquadest.

This difference in results could be due to P. gingivalis bacteria, a Gram-negative bacterium with a more complex structure. The antibacterial compounds of cocoa bean extract are challenging to diffuse into the bacterial cell membrane. In this study, the material used was a whole extract of cocoa bean extract without separating the active compounds. Other compounds that are thought to inhibit antibacterial activity are a weakness in using this whole extract. These other compounds will inhibit the penetration of the active compound into the bacterial cell wall[25]. Other compounds that can inhibit antibacterial activity, according to Ebi and Ofoefule (1997), are fat, wax, and chlorophyll[26]. According to Sulistyowati and Soenaryo (1988) and Yusianto (1997), the fat content of the unfermented cocoa bean extract was 0.07% to 5.69% lower than the fermented one[27]. According to BSN (2008), cocoa beans have a fat content of seeds of less than 56%. More fat content can inhibit antibacterial activity. As an antibacterial agent, it must also reach an adequate concentration to have a therapeutic effect to kill or inhibit the growth of bacteria[2].

The concentration of unfermented cocoa bean extract as an antibacterial against P. gingivalis had a more potent inhibitory concentration than fermented cocoa bean extract. At a concentration of 2%, unfermented bean extract has been able to act as an antibacterial. In comparison, the fermented cocoa bean extract can only act as an antibacterial at a concentration of 3%. So the minimum inhibitory concentration of unfermented cocoa bean extract is 2%, while the fermented cocoa bean extract is 3%. Minimum inhibitory concentration (MIC) is the lowest concentration of antibiotics that can still inhibit the growth of certain organisms. The difference in the minimum inhibitory concentration occurred because the content of active compounds in the unfermented cocoa bean extract was more than the fermented one. Fermentation can cause a decrease in the content of active compounds in cocoa beans[28].

According to Puerto's (2016) research, during the fermentation process, the results showed decreased polyphenol content in cocoa beans. The polyphenol content decreased after fermentation due to the diffusion of polyphenols out of the cotyledons, besides polyphenols being oxidized and condensed[29, 30]. The decrease in the content of this active compound only occurs in non-fat ingredients so that the fat content will be relatively more. This is also influenced by the length of time the fermentation is carried out[27]. In the fermentation process, the sugar bioconversion in the mucus will become intermediate organic components such as ethanol, lactic acid, acetic acid, and other organic acids that will inhibit continuous seed germination. These spontaneous biochemical changes in the seeds also reduce the bitterness and astringency of the seeds. Several enzymatic reactions also occur that contribute to the formation of the desired taste and color of cocoa beans[31].

The main purpose of fermentation is to kill the seeds so that changes occur in the seeds, such as the color of the seed chips, the formation of aroma and flavor precursors, and facilitate the decomposition of the pulp. The fermentation process will change the color of the seeds from purple and solid gradually to brown and hollow. Cocoa beans that are not fermented (slaty) are dominantly gray in color, and cocoa beans that are purple in color indicate that the beans are not completely fermented, while dark brown and hollow cocoa beans indicate that the beans have been fermented entirely (fermented)[32]. The color of the cocoa bean is usually used as an indicator of the maturity level of the cocoa beans[31].

Based on previous research conducted by Wollgast and Anklam in 2000, the fermentation process can cause the active content such as polyphenols to decrease. In 2007, Caligianiet et al. proved this was due to the diffusion of polyphenols out of the cotyledons besides polyphenols being oxidized and condensed. The process of seed death causes cell permeability to be damaged so that polyphenol compounds come out of the cells and diffuse throughout the seed tissue. As a result, there will be a reaction with the polyphenol oxidase enzyme, which causes changes in polyphenol compounds[30]. The effect of fermentation is not only on the content of polyphenols but also on other content[33].

CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that the antibacterial activity of the unfermented Edel cocoa bean extract (*Theobroma cacao L.*) has antibacterial activity against P. gingivalis, which is stronger than the fermented one with a minimum inhibitory concentration of unfermented cocoa bean extract is 2%. In comparison, the fermented cocoa bean extract is 3%.

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