Antibiofilm effect of *Theobroma cacao* (cacao pod) extract on *Aggregatibacter actinomycetemcomitans* biofilm *in vitro*

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**Abstract**

Successful of periodontal treatment is to eradicate biofilm of bacteria. *Aggregatibacter actinomycetemcomitans* is a Gram-negative bacterium that have been suggested to be the main causes of periodontal disease. *Theobroma cacao* (cacao pod) is a medicinal plant that has a broad range of pharmacological effects. The aim of this study was to assess the antibiofilm effect of cacao pod extract against *A. actinomycetemcomitans* biofilm *in vitro*. *A. actinomycetemcomitans* were cultured in Brain Heart Infusion broth. Crystal-violet staining in biofilm assays were used to evaluate the cacao pod extract effect on *A. actinomycetemcomitans* ATCC 33384 biofilms and 0.2% chlorhexidine-gluconate was used as a positive control. After 24 hours of incubation, the optical density of each well in microtiter plates was measured. The results showed that the biofilm density after incubation with the cacao pod extract was significantly decreased in all concentrations and all incubation times (p<0.05). The most effective concentration for inhibiting biofilm *A. actinomycetemcomitans* was 100% cacao pod extract and 3 hrs of incubation time (p<0.05) with a 98.9% reduction of biofilm compared to negative control. Cacao pod extract is effective in inhibiting the growth of *A. actinomycetemcomitans* biofilm.

**Keywords:** *Aggregatibacter actinomycetemcomitans*, biofilm, cacao pods

**Introduction**

According to the World Health Organization (WHO), one of the main health issues in South East Asia is related to oral and dental health (WHO, 2009). The prevalence of oral and dental disease in South East Asia in 2009 was 32-37% (WHO, 2009). The most common oral and dental diseases are dental caries and periodontal disease. In particular, based on the ‘Survei Kesehatan Rumah Tangga’, the prevalence of periodontal disease in Indonesia is 60% (Badan Penelitian dan Pengembangan Kesehatan RI, 2009). This high prevalence was caused by a low societal awareness of dental and oral hygiene (NL, 2005).

Periodontal disease is a common infectious disease in the periodontal structure and is related to Gram-negative bacteria (Tampubolon, 2006). The most common diseases on periodontal tissue are gingivitis and periodontitis (Haynes *et al*., 2014). A healthy periodontium tissue includes Gram-positive bacteria, such as *Streptococcus anginosis* and *Actinomyces naeslundii* (Perry, 2014). In poor oral hygiene, Gram-negative...
bacteria, such as *P. gingivalis, Campylobacter* spp., *Tannerella forsythia, Treponema denticola* and *A. actinomycetemcomitans*, will increase and contribute to periodontal inflammation (Duerden, 1991; Radita *et al.*, 2019; Widyarman *et al.*, 2018). *A. actinomycetemcomitans* is a main etiology in periodontitis (Mättö *et al.*, 1997) and also an obligate anaerobic Gram-negative bacterium (Fine *et al.*, 2007; Perace *et al.*, 1996).

During a caries activity, the bacterial product interacts with gingival epithelial and penetrates into the fibroblast, periodontal ligament and alveolar bone (Lafaurie *et al.*, 2007; Setiawati, 2012). The bacteria attach to one another on a solid surface coated by the matrix, which consists of polysaccharide, extracellular DNA (eDNA) and protein, known as biofilm (Wei *et al.*, 2013). Biofilm can be a main etiology of virulence factors in tooth damage, periodontal disease and systemic disease because of the pathogenic bacteria in oral biofilm (Li *et al.*, 2000). During a caries activity, the bacterial product interacts with gingival epithelial, this is some examples of the pioneering bacteria that attach to dental surfaces and oral mucosa (Huang *et al.*, 2011).

There are some treatments for controlling oral hygiene, such as diet and plaque control, which balance the normal flora in the oral cavity. Plaque control can be performed mechanically and chemically. Mechanical plaque control can be performed using conventional methods, such as tooth brushing and dental flossing, however these methods are incapable of removing plaque accumulation on certain parts, such as the gingival sulcus (Cobb, 2008). The disadvantages of conventional methods can be overcome by combining them with chemical methods such as mouth wash (Wolf *et al.*, 2005). The chemical materials in mouth rinse are phenol, hexetidine, flour and chlorhexidine. The disadvantages of chemical materials for long-term use are tooth discoloration and allergies (Suhag *et al.*, 2007).

Contemporary society chooses to lead a natural lifestyle. This can be seen in the many uses of plants as medicines, which play an important role in life. WHO exemplifies this “back to nature” concept by recommending the use of traditional medicine to preserve health and prevent disease in society (WHO, 2005). Contemporary society chooses to lead a natural lifestyle. Phytopharmacology is a medicine originally made from natural ingredients, which has a certain utility (Dewoto, 2007).

One phytopharmacological material in Indonesia is cacao (*Theobroma cacao*). Cacao production in Indonesia reaches the third largest in the world (Pusat Penelitian Kopi dan Kakao Indonesia, 2010). Cacao produces seeds and releases 75% of wastes, such as the cacao pod (Figure 1). This large amount of cacao pod waste becomes a problem for the environment (Sartini *et al.*, 2012). Cacao pods consist of flavonoids, such as anthocyanin, catechin and leucoanthocyanidine, which are bioactive compounds that have antibacterial agent (Muliyatni *et al.*, 2012). Moreover, other compounds in the cacao pod are pectin and lignin. These compounds can potentially be developed into alternative medicines, food supplements and even cosmetics (Armiati *et al.*, 2016; Hii *et al.*, 2009).

![Figure 1. Theobroma cacao](image)
Previous studies have suggested that antibacterial activity on the cacao pod extract used on *Streptococcus mutans*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* has shown cacao pod extract potential for inhibiting bacterial growth (Monty, 2006). Cytotoxicity test results on cacao pod extract showed that the cacao pod is nontoxic (Yuanita, 2017). Cocoa has significant antibacterial effects against periodontal pathogenic bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia* (Hirao et al., 2010). Consuming a cocoa-enriched diet could diminish periodontitis-induced oxidative stress (Tomofuji et al., 2009). A research on cocoa beans (*Theobroma cacao* L) extracted with the ethanol 70% shows a higher antimicrobial activity against *A. actinomyctecemcomitans* than with water (Atikah et al., 2016).

There are many studies regarding the antibacterial effectiveness of cacao pod extract towards bacterial growth, but there is no research on the anti-biofilm activity in cacao pod extract towards *A. actinomyctecemcomitans*. Therefore, anti-biofilm activity tests of cacao pod extract towards *A. actinomyctecemcomitans* (the etiology bacteria in periodontal disease) are needed. Indonesian people are expected to use scientifically tested natural ingredients to prevent periodontal disease. The aim of this research is to analyze the ability of cacao pod extract to inhibit *A. actinomyctecemcomitans* biofilm growth in vitro.

**Materials and methods**

**Cacao extract preparation**

One kilogram of ripe cacao was taken from the tree, and the cacao pod was separated from the seed and placenta. The cacao pod was washed under running water, cut with a knife and dried in the sun. The dried cacao pod was blended and sifted using a 60-mesh strainer until the pod became powder and known as the sample. The purpose of making this cacao pod powder was to destruct the structural cell and tissue, thus the extract would be easily exposed to the solvent. Cacao pod extraction was performed using the maceration technique. In an Erlenmeyer tube, 40 g of cacao pod powder and 400 ml of 70% ethanol were mixed. The Erlenmeyer tube was inserted into a shaker and shaken at 120 rpm at room temperature for 3 hours and then left for 12–15 hours. Moreover, the solution was filtered with Whatman No. 41 filter paper (Merck, Darmstadt) until filtrate in solid residue form was obtained. Final the filtrate with ethanol was inserted into a rotary evaporator to vaporize the solvent, and the concentrated cacao pod extract was obtained. The concentrated cacao pod extract was diluted in concentrations of 6.25%, 12.5%, 25%, 50% and 100%.

**Phytochemical screening**

Qualitative phytochemical analysis was to identify active compound of the ethanol extracts of cacao pod. The extracts were tested for the presence of alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

**Test for alkaloid**

For Mayer’s test, cacao pod extract was mixed with Mayer’s reagent (potassium mercuric iodide solution). The creamish color precipitate was formed, indicate the presence of alkaloids.

**Test for saponin**

For foam test, 1 g powder was mixed with 5 mL of distilled water and shaken for 10 min, Appearance of foam indicate the presence of saponins.

**Test of tannins and phenols**

For ferric chloride test, the crude extract was mixed with ferric chloride reagent (FeCl₃). Blue green colour appeared the presence of tannins.

**Test flavonoids**
For Shinoda test, cacao pod crude extract was mixed with a few of magnesium ribbon and hydrochloric acid. Occurrence of a pink, orange or red coloration indicate the presence of flavonoids.

Test for steroid and triterpenoid

For Liebermann-Burchard test, cacao pod crude extract was mixed with acetic anhydride boiled and cooled, few drops of \(\text{H}_2\text{SO}_4\) were added down from the side of the test tube. Blue green ring which showed the presence of steroid and the formation red colour indicate the presence of triterpenoids.

Test for glycoside

For Salkowski’s Test, cacao pod crude extract was mixed with chloroform, then added concentrated sulfuric acid were added and shaken. Brown red colour indicate the presence of glycosides.

**A. actinomycetemcomitans culture**

A. actinomycetemcomitans ATCC 33384 is an obligate anaerobic Gram-negative bacterium. The bacterial culture was performed in an anaerobic atmosphere (5% \(\text{CO}_2\)) on AaGM (\textit{A. actinomycetemcomitans} growth medium) in a petri dish and incubated for 24 hours at 37°C.

**Biofilm assay**

The \textit{A. actinomycetemcomitans} culture was transferred to 25 ml of Brain Heart Infusion broth (Oxoid, Hampshire, UK) and incubated for 24 hours at 37°C in an anaerobic atmosphere. The culture was then homogenized with a vortexer, and the bacterial colony growth was measured with a 450 nm wavelength. Optical density was measured with a microplate reader (SAFAS MP96, SAFAS, Monaco), and the result was OD 0.132 (1x10^7 CFU/ml). A 200 µL culture was distributed into 96 well-plate microplates and incubated at 37°C for 48 hours in an anaerobic atmosphere to form the biofilm. The supernatant was removed, and the well was rinsed twice with 200 µL Phosphate Buffered Saline (PBS). 200 µL of cacao pod extract in different concentrations (100%, 50%, 25%, 12.5% and 6.25%) were distributed into biofilm containing plates. The extract was incubated at 37°C for 15 min, 1 hour, 3 hours, or 6 hours in an anaerobic atmosphere, and the inhibition effect was observed. The supernatant was removed, and the well was rinsed twice with 200 µL PBS. Crystal violet (0.5% w/v) was distributed into the well-plate and incubated for 15 min. The extraction from violet crystal in the well-plate was measured as a biofilm number with the addition of 200 µL of ethanol absolute (Merck, Darmstadt) for 15 min, and the absorbance was measured with 490 nm wavelength. The biofilm without the cacao pod extract was used as a negative control, and 0.2% chlorhexidine was used as a positive control. All treatments were done in triplicate.

**Data analysis**

The data was analyzed using a parametric test method with the Saphiro-Wilk normality test. An ANOVA one-way statistical test was performed for normally distributed data. A Tukey-HSD test was performed to determine the significant differences. p<0.05 was considered significant.

**Results**

**Preliminary qualitative phytochemical screening analysis**

The present study's findings demonstrate that the ethanol extract of cacao pod extract contained alkaloids, saponins, phenolics, flavonoids, triterpenoids, and glycosides, but not tannins or steroids (Table 1).

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Results</th>
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<tr>
<td>Alkaloid</td>
<td>+</td>
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<tr>
<td>Saponin</td>
<td>+</td>
</tr>
<tr>
<td>Tanin</td>
<td>-</td>
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<tr>
<td>Phenolics</td>
<td>+</td>
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<tr>
<td>Flavonoid</td>
<td>+</td>
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<td>Triterpenoid</td>
<td>+</td>
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<tr>
<td>Steroid</td>
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<td>Glycosides</td>
<td>+</td>
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**The inhibitory effects of cacao pod extract against biofilm formation**

The results showed that the biofilm density after incubation with the cacao pod extract was significantly decreased in all concentrations and all incubation times \((p<0.05)\). The most effective concentration for inhibiting biofilm *A. actinomycetemcomitans* was 100% cacao pod extract and 3 hours of incubation time \((p<0.05)\) with a 98.9% reduction of biofilm compared to negative control (Figure 2-5).

Figure 2. The reduction graphic of *A. actinomycetemcomitans* biofilms (as measured by optical density) after 15 minutes application of cacao pod extract (6.25%, 12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control. All treatments were done in triplicate. (*\(p<0.05\) compared to the negative control)

Figure 3. The reduction graphic of *A. actinomycetemcomitans* biofilms (as measured by optical density) after 1-hour application of cacao pod extract (6.25%, 12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control. All treatments were done in triplicate. (*\(p<0.05\) compared to the negative control)
Figure 4. The reduction graphic of *A. actinomyetemcomitans* biofilms (as measured by optical density) after 3-hour application of cacao pod extract (6.25%, 12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control. All treatments were done in triplicate. (*p < 0.05 compared to the negative control)

Figure 5. The reduction graphic of *A. actinomyetemcomitans* biofilms (as measured by optical density) after 6-hour application of cacao pod extract (6.25%, 12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control. All treatments were done in triplicate. (*p < 0.05 compared to the negative control)

**Discussion**

Biofilm-associated periodontitis disease is a major cause of tooth loss in oral cavity dental cases. The primary etiologies factor is bacterial form microcolonies known as biofilm. Biofilms were found to protect from harm environmental and low nutrient condition (Berezow & Darveau, 2012). Choosing appropriate treatment is the important factor of successful in periodontitis treatment. Beside scaling and root planning as the primary therapy, use antiseptics therapy is the common choice for treating periodontitis.
Many of studies on the efficacy of synthetic medicine such as antiseptics have been established. Antiseptics are often used as adjunctive medicine with scaling and root planing. One of the antiseptics material for periodontitis treatment is chlorhexidine (CHX), CHX has been known as the gold standard of oral antiseptics (Mathur et al., 2011). Studies reported that use of 0.2% chlorhexidine had antibacterial effects on A. actinomycetemcomitans (Kadkhoda et al., 2016). CHX was also more effective than minocycline at killing P. gingivalis biofilm in vitro (Noiri et al., 2003). The mechanism of action of CHX is to destroying bacterial proteins and cell wall (De Wall et al., 2013). But there are some side effects of CHX, such as discoloration of teeth, tongue (distorted taste), allergic and cytotoxic effect. In vitro study of CHX to gingival cells showed that the toxic potency of CHX (Babic et al., 1995). Various allergic reaction due to CHX have been showed such as desquamative gingivitis, dermatitis, urticaria and occupational asthma (Dukes, 1992; Krauthheim, 2004).

Herbal medicines are generally considered to be safe and effective compare to synthetic medicines. About 8% of hospital admission in USA are due to adverse effects of synthetic drugs. At least 100,000 people each year die due to toxicities effect of synthetic drugs (Philomena, 2011). Herbal medicines contain various of active compounds that useful for health such as antioxidant activities, antibacterial, anti-inflammation and anti-cancer (Rafieian-Kopaie et al., 2011; Shirzad et al., 2011). One of the herbal medicines is cacao pod. The cacao pod has anti-inflammatory, antioxidant and antimicrobial material. The antimicrobial compound in cacao pods is flavonoid. The flavonoids in cacao pods are catechin, anthocyanin and leucaanthocyanidine (Mulyatni et al., 2012). Flavonoids are phenolic compounds and the result of phenylpropanoid cycle synthesis as a response against microbial infection. Flavonoids work by reducing the fluidity of the bacterial cell membrane. The flavonoids in the cacao pod have been proven to inhibit Streptococcus mutans, Escherichia coli, Bacillus subtilis and Staphylococcus aureus (Hii et al., 2009). The cacao pod is nontoxic (Armiati et al., 2016), and other compounds that can be found in the cacao pod include pectin and lignin (Hii et al., 2009).

Biofilm assay will provide information about the quantification of biofilm bacteria. This colorimetric assay is a semi-quantitative method based on dye (crystal violet) uptake by the bacteria cell in a biofilm. The biofilm assay analysis of this research is supported by other research which states that dried ethanol cacao pod extract with a 20% concentration has antibacterial effects against Escherichia coli, Salmonella typhosa, Staphylococcus aureus and Streptococcus mutans. The inhibition zones were 8.15 mm, 8.25 mm, 9.15 mm and 8.95 mm, respectively. Other research has shown that the potential of cacao pod extract to inhibit bacterial growth in the urinary tract and that the higher the extract concentration, the larger the inhibition zone against bacterial growth. The largest inhibition zone of Staphylococcus aureus and Escherichia coli growth was formed at a 64% concentration, and the result was 10 mm and 8.83 mm, respectively (Mulyatni et al., 2012).

Other research has shown that there was a reduction in optical density (OD) in 4 hours and 24 hours of incubation time at a 30% concentration against Streptococcus mutans, and this research also showed that cacao pod extract can reduce the acid production of Streptococcus mutans (Monty, 2006). Research by Yuanita et al. in 2017 stated that cacao pod extract in 100%, 50%, 25%, 12.5%, 6.25% and 3.25 % concentrations was able to reduce the optical density of Enterococcus faecalis biofilm; however, a concentration lower than a 3.25% was unable to reduce the density of Enterococcus faecalis (Yuanita, 2017).

Based on phytochemical tests at the Laboratorium Balai Penelitian Tanaman Rempah dan Obat (BALITRO), cacao pod extract consists of flavonoid, anthocyanin, catechins, saponin, lignin, pectin and triterpenoid. Flavonoid compounds consist of polyphenol with a benzo-y-pyrene chemical structure. Flavonoid has the ability, as an antibacterial, to reduce bacterial
enzymes, interfering with protein transport in the bacterial cell membrane and destroying the bacterial cell membrane (Chandki et al., 2011; Rose et al., 2004).

The glycoside on cacao pod extract is saponin. Saponins are able to destroy the cytoplasm membrane and affect the permeability of the bacterial cell membrane, so the exchange of material inside or outside is uncontrollable. Saponin also has a pharmacological role as a cough suppressant, an anti-inflammatory, a vasoprotective, a hipocolestrolemic, an immune-modulator, an antifungal and an anti-parasite (Ahman, 2017; Podolak et al., 2010). Anthocyanin is a coloring compound in the cacao pod. Catechins are compounds that act as antioxidants, and lignin and pectin are the main components of cell and structural composition in cacao pod tissue (Karatan et al., 2009). Another compound in the cacao pod is triterpenoid, which is a hydrocarbon component (C_{25}H_{46}) that gives a distinctive odor to some parts of the plant, such as the flower, fruit, leaf and branch. Triterpenoid also has an antimicrobial agent (Sawai et al., 2011).

With these results herbal medicines have potential as antibiofilm agents and many people every year turn to herbal medicines from synthetic medicines because they believe herbal medicines have low side effects (Kazemipoor et al., 2012). Many studies investigated antibiofilm effects of herbal products on bacterial biofilm suggesting their ability as alternative agents for bacterial infection.

**Conclusion**

Based on this research, it can be concluded that cacao pod extract has an inhibitory effect on the growth of *A. actinomycetemcomitans* biofilm and prevents periodontal disease, especially aggressive periodontitis. The higher the concentration of cacao pod extract, the greater the inhibitory effect on the biofilm viability of *A. actinomycetemcomitans*. Further study to observe cacao pod use as an effective anti-biofilm in the oral cavity is still needed. In vivo research is warranted to observe the side effects of cacao pod use.

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**References**


