

THE EFFECT OF *CURCUMA XANTHORRHIZA* ETHANOL EXTRACT ON THE VIABILITY OF *STREPTOCOCCUS MUTANS* AND *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* (DENTAL BIOFILM RESEARCH: *IN VITRO* STUDY)

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ABSTRACT

Objective: This study aimed to compare the effect of *Curcuma xanthorrhiza* ethanol extract to the viability of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* using single- and dual-species biofilm at different phases of formation.

Methods: Biofilm models were incubated for 4, 12, and 24 hrs, then exposed to the extract at a concentration of 0.525%.

Results: The viability of the single-species *S. mutans* biofilm was low ($p < 0.05$), and no significant difference ($p > 0.05$) was found between single-species *A. actinomycetemcomitans* and dual-species biofilm.

Conclusions: *Curcuma xanthorrhiza* ethanol extract is more effective for decreasing the viability of single-species *S. mutans* biofilm.

Keywords: *Aggregatibacter actinomycetemcomitans*, Biofilm, *Curcuma xanthorrhiza* ethanol extract, *Streptococcus mutans*, Viability.

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INTRODUCTION

The number of Indonesians experiencing oral and dental health problems increased from 2007 to 2013. According to the Basic Health Research (Riset Kesehatan Dasar), the percentage of the Indonesian population with oral and dental health problems was 23.3% in 2007 and increased to 25.9% in 2013 [1]. Of all oral and dental diseases, dental caries, and periodontal disease were found most often found in this population [2]. According to the WHO, 60-90% of the global population, including children and adults, had experienced dental caries. In Indonesia, according to the Basic Health Research (Riset Kesehatan Dasar), the decay-missing-filling-teeth index was 4.6 in 2013 [1,3]. Based on this data, it can be concluded that preventive measures against oral and dental health problems in Indonesia have not succeeded.

One way to prevent dental caries and periodontal disease is by hindering the role of bacteria, which can cause those diseases at a later stage of its development. Biofilm is a bacterial community consisting of various species (multispecies) that forms on a hard surface layered with a polysaccharide matrix. The formation of biofilm begins with bacterial adhesion to a hard surface, followed by further colonization of other bacteria and maturation in the presence of an exopolysaccharide matrix. This matrix can hinder the penetration of external substances into the biofilm, including antibiotics. As a result, microorganisms producing biofilm become more resistant to antibiotics and other antimicrobial substances [4,5].

Dental caries is a bacterial infection occurring on the hard tissue of the teeth, and it is a serious health problem due to its high incidence rate [1,3]. One type of bacteria causing dental caries is *Streptococcus mutans*, which produces acid that can lower oral pH to a critical level (< 5.5), causing demineralization and the formation of cavities. Moreover, it can initiate the formation of biofilm on the surface of teeth [5]. Periodontal disease is infection of periodontal tissue by periodontal pathogen bacteria in the form of biofilm. Further destruction of periodontal tissue can result in the destruction of the supporting alveolar bone, tooth mobility, and tooth loss [6,7].

One type of periodontal pathogen bacteria is *Aggregatibacter actinomycetemcomitans*, a Gram-negative anaerobic occurs bacterium. *A. actinomycetemcomitans* in the form of biofilm can shield the bacteria from its host's immune response, which involves the continuous release of inflammatory mediators and causes tissue destruction. *A. actinomycetemcomitans* is also the main type of bacteria causing aggressive periodontal disease, which is characterized by the destruction of periodontal tissue and alveolar bone resorption over a short period. *A. actinomycetemcomitans* is also related to other infectious diseases that occur outside the oral cavity, such as endocarditis, meningitis, osteomyelitis, and brain abscess [7-9].

Currently, there is a trend in Indonesia to use herbal medications derived from plants in an attempt to go "back to nature [10]." These herbal medicines are considered safer than synthetic drugs because they have fewer side effects and are less expensive. Often, the parts of a plant with medicinal properties are the secondary metabolites. Indonesia, which has the most biological diversity in the world after Brazil, is estimated to have 7,000 species of plants with medical properties [11,12]. Indonesians have utilized herbal medicines for a long time, and knowledge about how to prevent and cure diseases and use plants for cosmetic purposes is passed down from generation to generation [12,13]. To develop herbal medicines in Indonesia, the Drug and Food Control Agency (Badan Pengendalian Obat dan Makanan) of the Republic of Indonesia identified nine types of superior plants with medicinal properties, including curcuma [11,14].

Curcuma (*Curcuma xanthorrhiza* Roxb.) is a medicinal plant originating from Indonesia. This plant can help resolve hepatic problems, rheumatism, and fatigue, and it has analgesic, antibacterial, antifungal, antidiabetic, antioxidant, antidiarrheal, antitumor, and antidepressant functions. The rhizome is the most commonly used part of the curcuma plant because it has a high starch content (41.45%), and thus has the potential to be integrated into food ingredients.

The plant also contains active substances, such as xanthorrhizol [14,15]. This substance can function as an antimetastatic, anticancer, and

anti-inflammation medication [16]. Furthermore, xanthorrhizol has an antibacterial effect on several oral pathogenic bacteria. One study found that xanthorrhizol is a strong bactericide, hinders acidogenesis, and can change the microbiobiofilm structure of *S. mutans* [17]. It can be found in the ethanol extract of curcuma, along with other active compounds in the form of curcuminoids, such as terpenoid, phenol, flavonoid, saponin, glycoside, alkaloid, and coumarin [18].

The extract can be thin or thick depending on the solvent used to perform the extraction. The method used in this study was chosen because it is simple and inexpensive. Ethanol was chosen as a solvent due to its high polarity, which provides it a stronger ability to extract curcuma substances. The general objective of this study was to compare the viability of *S. mutans* and *A. actinomycetemcomitans* during various phases of the formation of single- and dual-species biofilm after exposure to curcuma ethanol extract.

METHODS

The curcuma ethanol extract used in this study was processed by BALITRO, Bogor. Ethanol extract was centrifuged at 3,000 rpm for 20 minutes until four layers were obtained. Only the first layer was used. The extract was then diluted to concentrations of 0.5%, 1%, 5%, 10%, 15%, 20%, and 25% using dimethyl sulfoxide 10% solvent.

Biofilm models were divided into three groups: Single-species *S. mutans*, single-species *A. actinomycetemcomitans*, and dual-species *S. mutans* and *A. actinomycetemcomitans*. Each group was accompanied by negative and positive controls. The negative control was a biofilm model that was not exposed to any test substance, whereas the positive control was a biofilm model that was exposed to 0.2% chlorhexidine.

The single-species biofilm model was made by mixing bacterial suspension into BHI broth and adding 0.2% sucrose (w/v). Then, 100 µl of this mixture was placed in each well of a 96-well plate. Each well plate was filled with 100 µl of broth media with 0.2% sucrose (w/v). The well plate was then incubated for 4 hrs (biofilm adhesion phase), 12 hrs (biofilm active accumulation phase), or 24 hrs (biofilm maturation phase) in anaerobic conditions at 37°C so that a biofilm formed. For single-species *A. actinomycetemcomitans* biofilm and dual-species *S. mutans* and *A. actinomycetemcomitans*, the BHI broth was enriched with Vitamin K. Wells in which biofilms had formed and that had been rinsed once with sterile phosphate-buffered saline were exposed to 100 µl of curcuma ethanol extract at concentrations of 0.5%, 1%, 5%, 10%, 15%, 20%, and 25% as well as blank wells. For the negative controls, 100 µl of BHI broth media was placed in wells in which biofilms had formed, including blank wells. For the positive controls, 100 µl of 0.2% chlorhexidine was placed in wells in which biofilms had formed, including blank wells. The well plates were then incubated for 60 minutes in anaerobic conditions at 37°C.

For an MTT test, 10 µl of MTT solution at a concentration of 5 mg/ml was placed in each well of a 96-well plate containing biofilms exposed to curcuma ethanol extract and wells that were used as positive controls, negative controls, and blanks. The well plate was then incubated in anaerobic conditions for 3 hrs at 37°C. After the incubation period passed, 100 µl of acidified isopropanol solution was placed in each well. The well plate was then shaken on an orbital shaker for 1 hr. Optical density score was determined using a microplate reader at a wavelength of 490 nm.

RESULTS

Fig. 1 shows the mean viability of single-species *S. mutans* biofilm after exposure to various concentrations of curcuma ethanol extract.

During the adhesion phase (4 hrs), the viability of biofilm was lowest when exposed to 10% curcuma ethanol extract (4.00±0.22%). During the active accumulation phase (12 hrs), the viability of biofilm was lowest when exposed to 15% curcuma ethanol extract (5.38±0.68%). Similarly, during the maturation phase (24 hrs), the viability of

biofilm was lowest when exposed to 15% curcuma ethanol extract (10.78±1.89%). At those three concentrations, the viability of biofilm was still higher than that of the positive control (0.2% chlorhexidine) during each phase of biofilm formation. Based on a *post hoc* analysis and one-way ANOVA test, these three viability scores were significantly different from that of the positive control ($p < 0.05$).

To assess the relation between increases in concentration of curcuma ethanol extract used and *S. mutans* biofilm viability, correlation Pearson test was used, which showed significant correlation ($p < 0.025$) between two variables in all biofilm formation phase. Furthermore, according to the regression analysis, the longer biofilm incubation time, its viability increases.

As shown in Fig. 2, exposure of curcuma ethanol extract to the viability of single-species *A. actinomycetemcomitans* biofilm was inconsistent with an increased concentration of curcuma ethanol extract. During the adhesion phase (4 hrs), the viability of biofilm was lowest when exposed to 1% curcuma ethanol extract (29.24±2.28%). During the active accumulation phase (12 hrs), the viability of biofilm was lowest when exposed to 20% curcuma ethanol extract (51.09±3.32%). During the maturation phase (24 hrs), the viability of biofilm was lowest when exposed to 5% curcuma ethanol extract (70.85±0.64%). At all three concentrations, the viability of biofilm was still higher than the positive control (0.2% chlorhexidine) during each phase of biofilm formation. Based on a *post hoc* analysis and one-way ANOVA test, these three viability scores were significantly different from that of the positive control ($p < 0.05$).

To assess the relation between increases in the concentration of the curcuma ethanol extract and the viability of *A. actinomycetemcomitans* biofilm, a correlation Pearson test was performed. The test showed significant correlation ($p < 0.025$) between two variables in the adhesion phase, with correlation coefficient (r) = 0.584. Meanwhile, during the active accumulation and maturation phases, the correlation between the two variables was not significantly different ($p > 0.025$). Furthermore, according to regression analysis, the longer biofilm is incubated, the higher its viability.

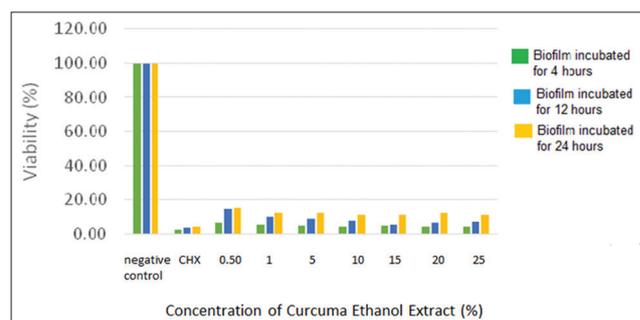


Fig. 1: Mean viability percentage of single-species *Streptococcus mutans* biofilm

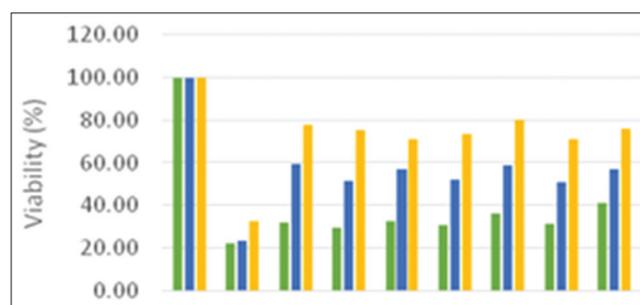


Fig. 2: Mean viability percentage of single-species *Aggregatibacter actinomycetemcomitans* biofilm

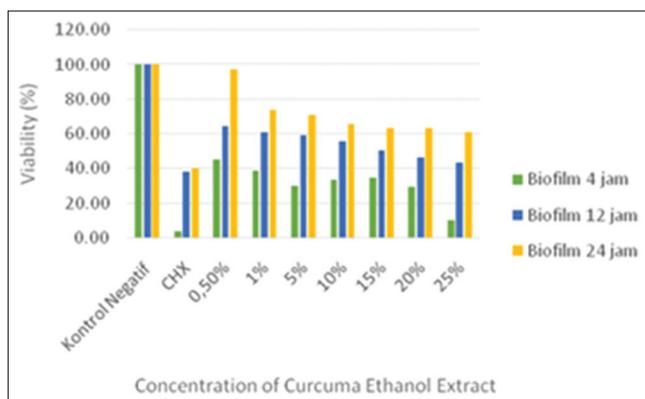


Fig. 3: Mean viability percentage of dual-species *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans*

Based on Fig. 3, the viability of dual-species *S. mutans* and *A. actinomycetemcomitans* biofilm exposed to curcuma ethanol extract decreased with increased concentrations of curcuma ethanol extract. During the adhesion phase (4 hrs), the viability of biofilm was lowest when exposed to 25% curcuma ethanol extract ($9.69 \pm 0.31\%$). Similarly, during the active accumulation phase (12 hrs) and the maturation phase (24 hrs), the viability of biofilm was lowest when exposed to 25% curcuma ethanol extract ($43.28 \pm 2.07\%$ and $61.05 \pm 0.82\%$, respectively). At all three concentrations, the viability of biofilm was still higher than that of the positive control (0.2% chlorhexidine) during each phase of biofilm formation. Based on a *post hoc* analysis and one-way ANOVA test, the viability of biofilm exposed to 25% curcuma ethanol extract during the adhesion and active accumulation phases was not significantly different from that of the positive control ($p > 0.05$). However, during the maturation phase, there was a significant difference between the viability of biofilm exposed to 25% curcuma ethanol extract and the biofilm viability of the positive control ($p < 0.05$). To assess the relation between an increase in the concentration of curcuma ethanol extract and the viability of dual-species *S. mutans* and *A. actinomycetemcomitans* biofilm, a correlation Pearson test was used. The test revealed significant correlation ($p < 0.025$) between the two variables during all phases of biofilm formation.

DISCUSSION

Exposure of curcuma ethanol extracts to *S. mutans* caused a decrease in biofilm viability along with increased concentrations of curcuma ethanol extract. *S. mutans* was found to be sensitive to curcuma ethanol extract. This sensitivity was caused by an active compound of xanthorrhizol in curcuma ethanol extract effect to peptidoglycan in *S. mutans* cell membranes. The phenol component of xanthorrhizol can cause protein coagulation and lysis of bacterial cell membranes. Cell lysis occurs as a result of hydrogen bonds between the hydroxyl group in phenol and a protein in the bacterial cell membrane that cause unequal cell membrane permeability, leaking of intracellular material, and cell death. The simple cell membrane structure of Gram-positive bacteria, including *S. mutans*, facilitates penetration of curcuma ethanol extract through cell membrane, disturbing cell biosynthesis [19].

In general, based on a correlation statistical analysis, an increase in the concentration of curcuma ethanol extract was followed by a decrease in the viability of *S. mutans* biofilm during all phases of biofilm formation (dose-dependent). During the maturation phase, *S. mutans* biofilm was more resistant than other biofilms during the adhesion and active accumulation phases because in the maturation phase, biofilm has an extracellular matrix that increases the integrity of the biofilm structure and resists antimicrobial substance molecules [4]. Moreover, quorum sensing occurs, which is affected by the density of bacterial cells in the biofilm. Quorum sensing, an interaction mechanism between bacterial cells, can change the physiological activity of bacteria, thus changing its virulence [20].

Exposure of *A. actinomycetemcomitans* biofilm to curcuma ethanol extract did not show consistent decrease or increase in viability. Even though the optimal concentration of curcuma ethanol extract to lower biofilm viability could be determined during each biofilm formation phase, the viability at the optimal concentration was not significantly different than the viability of biofilm that was exposed to other concentrations of curcuma ethanol extract. Furthermore, the viability at the optimal concentration was still higher than that of the positive control biofilm. It was found that inconsistency in the viability of *A. actinomycetemcomitans* biofilm could result from the properties of the membrane of *A. actinomycetemcomitans*, a Gram-negative bacteria composed of a lipopolysaccharide and lipoprotein bonded to peptidoglycan. Lipopolysaccharide and peptidoglycan function as an immune system for bacterial cells by identifying foreign substances penetrating the cell, thus causing curcuma ethanol extract to be unable to penetrate the cell membranes of Gram-negative bacteria, including *A. actinomycetemcomitans*. Penetration of curcuma ethanol extract into Gram-negative bacteria is also hindered by the presence of a lipoprotein located in the outer membrane of bacterial cells that contain hydrophilic proteins called porin and hinders the extract from penetrating the cell due to its hydrophobic property [21]. Moreover, the morphology of the *A. actinomycetemcomitans* cell membrane consists of vesicle-like folds that can inhibit penetration of curcuma ethanol extract into the periplasmic space and sitoplasma of bacteria. Vesicles on the outer membrane of *A. actinomycetemcomitans* also contain protease, which can deactivate the active compound in the extract [22]. *A. actinomycetemcomitans* and other bacteria comprising biofilm are more resistant to antibiotics and other antibacterial compounds due to the presence of an extracellular matrix, which serves as a protective layer over the biofilm and aids absorption and storage of nutrition for biofilm growth, making the biofilm more resistant to harmful environment changes [23].

Based on a correlation test, there was no significant correlation between increases in the concentration of curcuma ethanol extract and decreases in the viability of *A. actinomycetemcomitans* biofilm. However, during the adhesion phase, there was a significant positive correlation between increases in the correlation of curcuma ethanol extract and increases in the viability of *A. actinomycetemcomitans* biofilm. Although a positive and significant correlation was identified, the viability of biofilm at each concentration of curcuma ethanol extract was not significantly different or higher than that of the positive control group.

Exposure of higher concentrations of curcuma ethanol extract to dual-species *S. mutans* and *A. actinomycetemcomitans* biofilm resulted in decreased viability. During all phases of biofilm formation, exposure to 25% curcuma ethanol extract resulted in the lowest biofilm viability. However, generally, the viability of dual-species biofilm was higher than that of single-species *S. mutans* biofilm when exposed to all concentrations of curcuma ethanol extract. When compared to the viability of single-species *A. actinomycetemcomitans* biofilm, the viability of dual-species biofilm was lower when exposed to all concentrations of curcuma ethanol extract. However, these differences were not statistically significant. Therefore, it could be concluded that although the viability of dual-species biofilm was lowered when exposed to curcuma ethanol extract, the viability of dual-species biofilm was still high compared to that of single-species *S. mutans* biofilm, which was considered to respond well to curcuma ethanol extract.

The high viability of bacterial cells in dual-species biofilm could be caused by interactions between bacteria of the same and different species in the biofilm, which change the virulence of the bacteria [23]. Moreover, *S. mutans* and *A. actinomycetemcomitans* have a synergistic relationship in which *S. mutans* provides energy to *A. actinomycetemcomitans* in the form of lactic acid, which is a metabolic product of sucrose in *S. mutans*. Although sucrose can be used by *A. actinomycetemcomitans* as an energy source, lactic acid is the main source of energy for this bacteria. When the bacteria derives energy from lactic acid, it can

become more competitive than when it derives energy from sucrose, such as when the bacteria is cultured in single-species biofilm so that it can grow over *S. mutans* [24]. Dual-species biofilm during the maturation phase was more resistant than dual-species during the adhesion and active accumulation phases. Due to the factors explained above, the extracellular matrix hinders ethanol extract molecules from penetrating the biofilm [23].

Comparison of these three biofilm model groups during three formation phases revealed that single-species *S. mutans* biofilm responded better to curcuma ethanol extract than single-species *A. actinomycetemcomitans* and dual-species biofilm. In addition, compared to dual-species biofilm, single-species *A. actinomycetemcomitans* biofilm did not respond differently to curcuma ethanol extract, especially in the adhesion and active accumulation phases. Single-species *S. mutans* biofilm was more sensitive than single-species *A. actinomycetemcomitans* biofilm due to differences in the cell membranes of these bacteria that facilitate penetration of xanthorrhizol into bacterial cells and cause leakage of intracellular material and cell death [17]. Single-species *S. mutans* biofilm was more sensitive than dual-species biofilm. This could be caused by differences in the composition of these biofilms; the more bacterial species are in a biofilm, the more complex the interactions not only between bacterial cells within the same species but also between different species. In biofilm, interactions between bacteria can be synergistic, cooperative, or competitive. Interactions between *S. mutans* and *A. actinomycetemcomitans* biofilm are competitive in an attempt to obtain nutrition and other factors that are important for growing and developing the structure of biofilm [20]. However, *S. mutans* and *A. actinomycetemcomitans* bacteria are also known to have a synergistic relationship in which *S. mutans* produces lactic acid through metabolic activity that can be used by *A. Actinomycetemcomitans* as a main source of energy [24]. This could explain why dual-species biofilm is more resistant than single-species *S. mutans* biofilm. Moreover, gene involved in resistance to antibacterial compounds could be transferred from one bacterial cell, such as *A. actinomycetemcomitans*, to another bacterial cell in the biofilm in this study, *S. mutans* so that the overall resistance of the biofilm was increased [24,25]. In addition, in dual-species biofilm, the curcuma ethanol extract may have been able to hinder the growth of *S. mutans* but not *A. actinomycetemcomitans* due to differences in the composition of the membrane cells of the two bacteria. Therefore, the viability of dual-species biofilm was high when measured with a microplate reader because the viability was actually due to *A. actinomycetemcomitans*. This possibility is supported by the fact that the viability of single-species *A. actinomycetemcomitans* biofilm and that of dual-species biofilm were not significantly different during the adhesion and active accumulation phases. However, it could not be conclusively determined since it was not known which bacteria dominate biofilm after exposure to curcuma ethanol extract. Further studies on the effectiveness of curcuma ethanol extract in regards to dual-species *S. mutans* and *A. actinomycetemcomitans* biofilm should use other methods, such as PCR, to identify which bacteria dominate biofilm after exposure to curcuma ethanol extract.

CONCLUSION

Curcuma ethanol extract can lower the viability of single-species *S. mutans*, single-species *A. actinomycetemcomitans*, and dual-species *S. mutans* and *A. actinomycetemcomitans* biofilm in all phases of formation. However, curcuma ethanol extract can more effectively lower the viability of single-species *S. mutans* biofilm compared to the other two biofilm groups, whereas the effectiveness of curcuma ethanol extract in regards to decrease the viability of single-species *A. actinomycetemcomitans* and dual-species biofilm is not very different.

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