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Research Article

EFFECT OF *CURCUMA XANTHORRHIZA* ROXB. ETHANOL EXTRACT ON THE VIABILITY OF *STREPTOCOCCUS MUTANS* AND *STREPTOCOCCUS SANGUINIS* DUAL-SPECIES BIOFILMS

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ABSTRACT

Objective: This study aimed to determine the effect of ethanol Curcuma extract on the viability of *S. mutans* and *Streptococcus sanguinis* in a dual-species *in vitro* biofilm model.

Methods: Dual-species biofilms of *S. mutans* and *S. sanguinis* were exposed to ethanol Curcuma extract at various concentrations. The sample of saliva was gathered from healthy volunteers. Chlorhexidine 0.2% was used as a positive control, and bacterial culture without intervention served as a negative control. The total suspensions of $10^{-\alpha\sigma}$ were prepared for *S. mutans* and *S. sanguinis* cells. The bacteria were incubated for 20 h (active maturation phase) and 24 h (maturation phase).

Results: The result showed decreased *S. mutans* and *S. sanguinis* viability after exposure to 0.2%–25% Curcuma ethanol extracts during the active accumulation and maturation phases. The decrease in bacterial viability was significantly different in all concentrations of Curcuma ethanol extracts compared with negative controls ($p<0.05$) in the active accumulation and maturation phases.

Conclusion: Temulawak ethanol extract (starting at 0.2%) can decrease the viability of *S. mutans* and *S. sanguinis* in a dual species *in vitro* biofilm model during the accumulation and maturation phases.

Keywords: *Dual* species, *Curuma xanthorriza ethanol extract*, *Streptococcus mutans*, *Streptococcus sanguinis, Viability*.

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INTRODUCTION

Dental caries is a disease of the tooth tissue, enamel, dentin, and cementum caused by the activity of microorganisms on fermented carbohydrates. The formation of caries is characterized by demineralization of tooth tissue, followed by the breakdown of organic components. Bacterial invasion of caries damages the pulp tissue and spreads the infection to the periapical tissue and cause pain [1]. According to Keyes and Jordan, three main factors contribute to caries: A host, a substrate (diet), and an agent (microorganism). Time (duration) is another important contributing factor [2,3]. Caries occurs when all three factors are present and work simultaneously over a period of time [1].

A biofilm is an aggregation of microorganisms attached to a surface and covered by extracellular polysaccharides that are a major component of biofilms and assist in maintaining biofilm structures [4]. There are various species within natural biofilms that communicate through quorum sensing and affect each other, either synergistically or antagonistically [5]. The formation of biofilms in the oral cavity can be divided into three phases: Adhesion, active accumulation, and maturation. The adhesion phase is the initial stage of biofilm formation involving an adhesive attachment, which is a thin protein film derived from salivary glycoproteins. The adhesion is related to the interaction between oral bacteria and the host molecules. The active accumulation phase occurs after the pioneer bacteria are attached, and the early colonizing bacteria (pioneer colonizers) provide a place for the next bacteria to colonize either directly or through salivary glycoproteins. Furthermore, colonializing bacteria recognize polysaccharides or receptor proteins on the surface of pioneer and attached bacterial cells. During the maturation phase, bacterial growth slows down or stops due to limited nutrients. The bacteria will leave the biofilm by separating from a single cell or a group of cells [6,7].

Bacterial competition is common in biofilms, in which these bacteria compete for nutrients, attachment, and survival. Certain bacteria within biofilms express nonspecific proteins (e.g., bacteriocin) that can affect other bacteria. Production of organic acids is one of the competitive mechanisms of *Streptococcus mutans* in inhibiting *Streptococcus sanguinis*. *S. mutans* produces more acid than *S. sanguinis* due to the high activity of ATP-glucose phosphotransferase in *S. mutans*. Therefore, lactic acid produced by *S. mutans* is excreted into the environment and inhibits the growth of *S. sanguinis*. Therefore, *S. mutans* increases in number compared with *S. sanguinis*. *S. mutans* also inhibits *S. sanguinis* with Mutasin I and IV. Mutasin can enhance the competitiveness of *S. mutans* in colonization and its ability to develop biofilms. In a third inhibitory mechanism, *S. mutans* inhibits the ability of *S. sanguinis* to produce hydrogen peroxide [4].

Primary prevention is a precautionary procedure before the onset of disease. A primary prevention measure against dental caries is using chlorhexidine as a mouthwash. Although chlorohexidine has broad and potent antimicrobial effects, it also has long-term side effects such as staining the teeth and disturbing the ecology of oral bacteria [8].

Curcuma xanthorrhiza Roxb. (temulawak) is a plant found in various parts of Indonesia that has been established by the Food and Drug Supervisory Agency (BADAN POM) as one of the leading medicinal plants [9,10]. *Curcuma* is also used an ingredient in almost all types of traditional medicine in Indonesia [11]. In general, using traditional medicine is considered safer than using synthetic drugs because traditional medicine has relatively fewer side effects than synthetic drugs [12]. The use of Curcuma has progressed, starting from the preparation of traditional medicine and developed into standardized herbal drugs, which can then become phytopharmaca preparations [11].

Curcuma rhizomes contain 48%–59.64% starch, 1.6%–2.2% curcumin, and 1.48%–1.63% essential oils [13]. These three components serve

as the most important secondary metabolites that are beneficial for health, in industry, and household [14,15]. In addition to these three components, Curcuma contains protein, fat (fixed oil), cellulose, and minerals [14]. Curcuma has also been widely reported to be useful as an antimicrobial and anti-inflammatory compound [13].

One of the active components of essential oils in Curcuma that has an antibacterial effect is xanthorrhizol. According to Hwang's research, isolation of xanthorrhizol from Curcuma rhizomes has a high anticarcinogenic activity against oral pathogens. Xanthorrhizol exhibits the highest antibacterial activity against *Streptococcus* species that cause dental caries and also exhibits antibacterial potency against *Actinomyces viscosus* and *Porphyromonas gingivalis*, which cause periodontitis. Moreover, xanthorrhizol acts as an antibacterial agent to inhibit and eliminate *S. mutans* biofilms [16]. This antibacterial activity is thought to arise from phenol compounds in the Curcuma extract. Hydrogen bonds form between hydroxyl groups in the phenol compounds with cell membrane proteins, disrupting membrane permeability, and resulting in cell death. The coagulation of proteins and lysis of cell membranes result from the penetration of high amounts of phenol into cells [17,18]. Previous studies have shown that xanthorrhizol can destroy the integrity of cell walls. Xanthorrhizol exposure damages the peptidoglycan layer of *S. mutans*, causing leakage of intracellular contents and subsequent cell death [19].

A study by Rahmawati at the Faculty of Dentistry Universitas Indonesia reported that the viability of *S. mutans* biofilms decreased with increasing concentrations of ethanol Curcuma extract during the active accumulation phase (20 h) and maturation phase (24 h) [20]. Similarly, a study conducted by Fidinina (2011) demonstrated that ethanol Curcuma extract at a concentration of 0.5%–5% can decrease *S. sanguinis* viability in both the active accumulation and the maturation phases [21].

Based on the results of previous research on the effect of ethanol extract on the viability of single-species biofilms, this study aimed to determine the effect of ethanol Curcuma extract on the viability of *S. mutans* and *S. sanguinis* in a dual species *in vitro* biofilm model. It is well known that when growing as a dual species biofilm, these two bacteria compete with each other due to the limited space and nutrient availability. There is also an antagonistic relationship between *S. mutans* and *S. sanguinis* when grown simultaneously.

METHODS

Dual species biofilms of *S. mutans* and *S. sanguinis* were exposed to ethanol Curcuma extract at various concentrations. Curcuma ethanol extract was obtained by the maceration method derived from the Indonesian Medicinal and Aromatic Crops Research Institute (BALLITRO), Bogor. Chlorhexidine 0.2% was used as a positive control, and bacterial culture without intervention served as a negative control. Suspensions of 10^{-5} *S. mutans* and *S. sanguinis* cells were prepared. To create the biofilm model, the first saliva coating was applied to 96-well plates to obtain the pellicle. Saliva was obtained from healthy volunteers. After pellicle formation, the plates were washed with as much as 100 µl of phosphate buffer solution (PBS), and suspensions of 50 µl of *S. mutans* and 50 µl of *S. sanguinis* were added. The bacteria were incubated for 20 h (active maturation phase) and 24 h (maturation phase).

Following incubation, 100 µl of Curcuma ethanol extract was added at 0.2%, 1%, 5%, 10%, 15%, 20%, and 25% concentrations. As a positive control, 100 µl of 0.2% chlorhexidine was used. As a negative control, *S. mutans* and *S. sanguinis* were not exposed to Curcuma ethanol extract or 0.2% chlorhexidine, but 100 µl of brain heart infusion broth was added. After 1 h of incubation, cells were washed with 100 µl of PBS (5 mg/ml) solution and incubated for 2.5 h. Acidified isopropanol solution (100 µl) was added to each well, and plates were placed on top of the orbital shaker for 1 h. Finally, the optical density (OD) values were read on a microplate reader at 490 nm.

Bacterial viability was analyzed by Shapiro–Wilk and Kruskal–Wallis tests. The Shapiro–Wilk test was used to test the normality of data distribution. Then, a non-parametric Kruskal–Wallis test was performed to determine whether there was a significant difference among treatment groups, and a *post hoc* analysis was performed with Mann–Whitney U-test.

RESULTS

The results of the MTT assay during the active accumulation phase (20 h) are presented in Table 1. MTT assay results during the maturation phase (24 h) are presented in Table 2. A summary of results during both phases are presented in Table 3.

The Kruskal–Wallis test showed decreased *S. mutans* and *S. sanguinis* viability after exposure to 0.2%–25% Curcuma ethanol extracts during the active accumulation and maturation phases. The decrease in bacterial viability was significantly different in all concentrations of Curcuma ethanol extracts compared with negative controls ($p<0.05$) in the active accumulation and maturation phases. Chlorhexidine (0.2%) as a positive control also significantly decreased the viability of the bacteria compared with the negative control. The effects of Curcuma ethanol extracts did not significantly differ from those of 0.2% chlorhexidine. In addition, bacterial viability in the maturation phase tended to be higher than that in the active accumulation phase. However, the Kruskal–Wallis test indicated that this result was not significant ($p=0.377$); hence, a *post hoc* test was not performed.

DISCUSSION

This study showed that the viability of the *S. mutans* and *S. sanguinis* dual species biofilm significantly decreased when exposed to Curcuma ethanol extract (0.2%) compared with negative control. There was an increase in the percentage of bacterial viability after exposure to Curcuma ethanol extract at concentrations of 1%, 5%, and 10%. However, the increased bacterial viability was still lower than that of the negative controls. These results showed that the effects of ethanol Curcuma extract on the viability of *S. mutans* and *S. sanguinis* are independent of their concentration. It is possible that the yellow color of a curcuminoid and high viscosity of Curcuma ethanol extracts can influence the viability of the dual species *Streptococcus* using the MTT test, thus causing inaccuracy in the reading of OD values. It is also possible that decreased viability of the dual species *Streptococcus* biofilm occurs only at low Curcuma concentrations.

The decrease in the viability of the dual species *Streptococcus* biofilm is likely due to the antibacterial activity of xanthorrhizol, which consists of phenol and hydrocarbon chains. The hydroxyl group is the main active antibacterial component. There are two mechanisms of anticaries metabolism of natural antimicrobial agents. The first mechanism is to destroy the integrity of the cell wall, and the second mechanism is to inhibit the process of bacterial adhesion in biofilm formation. Xanthorrhizol can destroy the integrity of cell walls. *S. mutans* exposed to Curcuma incurs damage to its peptidoglycan layer, causing leakage of intracellular contents and cell death.

The results of this study also showed that bacterial viability tended to decrease more during the active accumulation phase (20 h) than the maturation phase (24 h) when exposed to temulawak ethanol extract. This finding was expected because almost all antimicrobials are effective at killing rapidly growing cells (active accumulation phase). Slow growth during the maturation phase is a factor that causes cell resistance against antimicrobials from Curcuma ethanol extract.

This study also showed that there was no significant difference between the viability of dual species biofilms exposed to chlorhexidine 0.2% and those exposed to ethanol Curcuma extracts of various concentrations. This finding demonstrated that Curcuma ethanol extract has potential as an antibacterial compound against *S. mutans* and *S. sanguinis* dual species biofilms, even at a low concentration of 0.2%. However, the

Table 1: Effect of Curcuma ethanol extract on the viability of *Streptococcus mutans* and *Streptococcus sanguinis* during the active accumulation phase (20 h)

Concentration (%)	Viability (%)	p value to the negative control	p value to the positive control
Negative control (BHI broth)	100		0.037
0.20	18.049	0.037	0.127
1	31.788	0.037	0.050
5	41.494	0.037	0.050
10	49.787	0.037	0.050
15	33.315	0.037	0.050
20	28.856	0.037	0.050
25	28.572	0.037	0.127
Positive control (chlorhexidine 0.2)	3.664	0.037	

BHI: Brain heart infusion

Table 2: Effect of Curcuma ethanol extract on the viability of *Streptococcus mutans* and *Streptococcus sanguinis* during the maturation phase (24 h)

Concentration (%)	Viability (%)	p value to the negative control	p value to the positive control
Negative control (BHI broth)	100		0.037
0.20	24.409	0.037	0.050
1	28.925	0.037	0.050
5	45.198	0.037	0.127
10	60.646	0.037	0.050
15	59.535	0.037	0.050
20	41.793	0.037	0.513
25	33.262	0.037	0.513
Positive control (chlorhexidine 0.2)	7.671	0.037	

BHI: Brain heart infusion

Table 3: Effect of Curcuma ethanol extract on the viability of *Streptococcus mutans* and *Streptococcus sanguinis* during the active accumulation phase (20 h) and maturation phase (24 h)

Concentrations of extracts (%)	Viability at active accumulation phase (%)	Viability at maturation phase (%)
Negative control (BHI broth)	100	100
0.20	18.049	24.409
1	31.788	28.925
5	41.494	45.198
10	49.787	60.646
15	33.315	59.535
20	28.856	41.793
25	28.572	33.262
Positive control (chlorhexidine 0.2)	3.664	7.671

BHI: Brain heart infusion

antibacterial effect of curcuma extract at concentrations of 0.2%–25% did not show better effectiveness than 0.2% chlorhexidine. Findings were similar between the maturation phase (24 h) and the active accumulation phase.

CONCLUSION

We observed that temulawak ethanol extract (starting at 0.2%) can decrease the viability of *S. mutans* and *S. sanguinis* in a dual species *in vitro* biofilm model during the accumulation and maturation phases. The antibacterial effect of temulawak ethanol extract was effective during the active accumulation phase.

CONFLICTS OF INTEREST

None declared.

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