

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

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ABSTRACT

Objective: This *in vitro* study aimed to analyze the mass ratio of single- and dual-species *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* biofilm after exposure to *Curcuma xanthorrhiza* ethanol extract (Xan).

Methods: A bacterial suspension in brain heart infusion medium, enriched with 0.2% sucrose, was exposed to the Xan, incubated for 18 hrs, and analyzed using a crystal violet assay.

Results: This research concluded that the minimum inhibitory concentration of ethanol-temulawak extract against *S. mutans* was 5%, while the minimum bactericidal concentration was 15%.

Conclusions: Xan prevented biofilm formation of single-species *S. mutans* and dual-species *S. mutans* and *A. actinomycetemcomitans* more effectively than it did single-species *A. actinomycetemcomitans*.

Keywords: Biofilm formation, Single-species biofilm, Dual-species biofilm, *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Curcuma xanthorrhiza* ethanol extract, Crystal violet assay.

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INTRODUCTION

Dental and oral health problems, particularly caries and periodontal disease, are common in Indonesia. The Indonesia national health survey in 2013 results show prevalence at about 25.9% nationally, with 14 provinces having higher prevalence than that [1,2]. Dental caries and periodontal disease are caused by oral bacteria in biofilm form. *Streptococcus mutans* is the main etiological agent of dental caries, working as an early colonizer to start the formation of dental plaque [3,4]. The *Aggregatibacter actinomycetemcomitans* bacterium interacts with other bacteria in a biofilm to produce virulence factors and thus cause periodontal disease. Biofilm itself is a structure made up of an extracellular polymer matrix embedded with same- or mixed-species bacteria that form a community and adhere to the tooth surface. It is the main causative agent of caries and periodontal disease, as it is resistant to antimicrobial agents. Great importance is therefore given to finding a new antimicrobial agent to control biofilm [2,5].

The Indonesian government is currently developing the use of medicinal plants. The Indonesian Food and Drug Administration (BPOM) has highlighted nine as superior, one of them being temulawak, *Curcuma xanthorrhiza* (Xan). This plant, also called Java ginger and Javanese turmeric, contains Xan, an agent with a considerable antibacterial effect that can be used as an alternative herbal antibacterial agent. Its extract form is expected to maximize the effect of Xan and other active agents contained in the plant [6,7]. Because *S. mutans* and *A. actinomycetemcomitans* in the biofilm are primary bacterial agents of dental caries and periodontal disease, an examination of the effect of temulawak extract on biofilm formation would be a useful preventive approach. This study aimed to evaluate the effect of temulawak extract on single- and dual-species *S. mutans* and *A. actinomycetemcomitans* biofilm formation.

METHODS

The raw material for this experimental laboratory study was ethanol-temulawak extract obtained from BALITRO, Bogor. The extract was

centrifuged (at 3000 rpm for 20 minutes at 4°C) and the supernatant layer collected. The supernatant was diluted using 10% dimethyl sulfoxide to produce several final concentrations (0.5%; 1%; 5%; 10%; 15%; 20%; and 25%), determined by volume comparison formula. The bacterial culture was started by isolating 10 µl of each species. The bacteria were inoculated into brain heart infusion (BHI) medium and incubated in an anaerobic chamber for 2 × 24 hrs for *S. mutans* and 3 × 24 hrs for *A. actinomycetemcomitans*. From each inoculation set, one colony was collected to make aliquots. To decide the amount of bacteria to be tested, the aliquots were diluted in steps from 10⁻¹ to 10⁻⁸, and then, inoculated into BHI medium. The bacterial colony count identified the 10⁻⁵ suspension as the best for the experiment, as the colony numbers were high enough to give significant results while being low enough for accurate counting. To conduct minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, 100 µl volumes of the 10⁻⁵ bacterial suspension were transferred into each well of a 96-well plate before 100 µl of the different concentrations of ethanol-temulawak extract were added. The 96-well plate was then incubated in an anaerobic chamber for 48 hours. The MIC and MBC values were then calculated, using a formula modified from Quave, with ≥90% as MIC and ≥99% as MBC [8,9].

A BHI broth medium, 0.2% sucrose enriched, was prepared before the dilution of the bacteria. The bacteria were then diluted in the way described above, to make a 10⁻⁵ bacterial suspension. For *A. actinomycetemcomitans*, 1 ml of vitamin K was added to the 0.2% sucrose-enriched BHI broth. For each of three groups (treatment group, negative control group, and positive control group), 100 µl of 10⁻⁵ bacterial suspension in 0.2% sucrose-enriched BHI broth were put in each well. Each group was exposed to 100 µl of ethanol-temulawak extract or Xan in each of the various concentrations. The positive control group was exposed to 100 µl 0.2% chlorhexidine (CHX), the negative control group received 100 µl of 0.2% sucrose-enriched BHI broth, and the blank group contained 100 µl of 0.2% sucrose-enriched BHI broth and 100 µl of the tested material. For the dual-species bacterial suspension biofilm, 50 µl of *S. mutans* and 50 µl

of *A. actinomycetemcomitans* were added. This procedure used triplo. Afterward, the 96-well plates were incubated for 18 hours at 37°C in an anaerobic chamber.

After incubation, the liquids were aspirated and rinsed once with phosphate buffered saline (PBS) then fixed for 10 minutes before coloring with 0.5% crystal violet solution. The 0.5% crystal violet solution was prepared by mixing 250 µl of 100% crystal violet with 49.75ml of aquadest distilled water. Each well received 200 µl of crystal violet solution before a further incubation for 15 minutes at 37 °C. The crystal violet solution was then aspirated and the 96-well plate rinsed twice with sterile aquadest. Next, 200 µl of 96% ethanol were added to each well to dissolve the crystal violet before a third incubation, again for 15 minutes at 37°C. After the incubation, the optical density value was read with a microplate reader at a wavelength of 490 nm. Biofilm inhibition was determined if the value reached 50% or more [8]. Data analysis was performed using ANOVA for the differentiation test, followed by the Pearson correlation test; linear regression was used to analyze the relationship between independent variables (concentration of ethanol-temulawak extract) and dependent variables (biofilm formation inhibition value).

RESULTS

Before the biofilm model test was performed, a research had been conducted to determine the MIC and MBC of ethanol-temulawak extract on *S. mutans* and *A. actinomycetemcomitans*. The research reported that the MIC of ethanol-temulawak extract on *S. mutans* was 5%, MBC 15%. The MIC and MBC of ethanol-temulawak extract on *A. actinomycetemcomitans* were not determined because no inhibition percentage value on that species reached 90% (Table 1).

Table 2 shows that the inhibition values of ethanol-temulawak extract against formation of single-species *S. mutans* and dual-species *S. mutans/A. actinomycetemcomitans* biofilms were over 50% in all concentrations, but did not reach 50% against formation of single-species *A. actinomycetemcomitans* biofilm for any concentration. The

Table 1: Exposure of *S. mutans* and *A. actinomycetemcomitans* to ethanol-temulawak extract

Group	Inhibition (%)±SD <i>S. mutans</i>	Inhibition (%)±SD <i>A. actinomycetemcomitans</i>
Negative control	0	0
Positive control	100.19±0.94	100.28±0.26
0.5	85.85±1.20	83.44±9.20
1	88.78±1.30	81.86±6.27
5	91.14±1.42(KHM)	82.69±8.84
10	95.16±2.59	88.18±2.93
15	100.37±0.83(KBM)	82.59±7.42
20	100.04±0.96	83.90±3.89
25	101.62±1.96	83.60±6.90
10% DMSO solution	16.51±2.14	13.17±1.16

DMSO: Dimethyl sulfoxide, *S. mutans*: *Streptococcus mutans*

Table 2: The Effect of Ethanol-temulawak extract on single- and dual-species biofilm formation

Group	Inhibition (%)±SD single-species <i>S. mutans</i>	Inhibition (%)±SD single-species <i>A. actinomycetemcomitans</i>	Inhibition (%)±SD dual-species <i>S. mutans</i> and <i>A. actinomycetemcomitans</i>
Negative control	0.00	0.00	0.00
Positive control	110.53±11.28	70.23±0.88	110.85±3.10
0.5%	61.50±8.18	29.92±1.69	60.61±1.84
1%	61.88±6.83	33.86±0.91	64.03±3.13
5%	78.37±11.77	34.97±0.84	72.78±3.49
10%	90.91±1.50	43.25±4.65	79.82±1.84
15%	90.03±3.95	37.12±0.50	90.01±0.35
20%	87.81±2.86	43.92±2.85	85.70±1.07
25%	93.81±0.49	37.54±0.50	90.54±1.22

ANOVA test concluded that there was a significant difference ($p < 0.05$) between negative and positive control in all biofilm formation groups, at all concentrations. Fig. 1 demonstrates a tendency for higher concentrations of ethanol-temulawak extract to give higher inhibition values in all groups. This result was confirmed by the correlation and regression tests, which showed a strong positive correlation between inhibition value and concentration of ethanol-temulawak extract in all groups.

DISCUSSION

This research has revealed that ethanol-temulawak extract has an MIC of 5% and an MBC of 15% against *S. mutans*, but has no measurable MIC or MBC against *A. actinomycetemcomitans*. This is due to the different cell wall linings of the two bacteria. *S. mutans* is Gram-positive, with a simple cell wall lining, while *A. actinomycetemcomitans* is Gram-negative and has a more complex lining. As it has no outer layer, antimicrobial agents can permeate the *S. mutans* cell wall and cause biosynthesis disturbance. The hydroxyl group in the Xan contained in ethanol-temulawak extract contributes to antimicrobial activity by inducing cell wall protein denaturation; the protein of the cell leaks out and causes cell death [10,4]. In the Gram-negative *A. actinomycetemcomitans*; however, the outer layer consists of lipopolysaccharide, lipoprotein, and periplasm bound to peptidoglycan. The lipopolysaccharide functions as a defense system with the peptidoglycan and rejects foreign substances. Lipoprotein in the outer membrane of Gram-negative bacteria contains porin, hydrophilic protein, and it is very possible that it makes the cell impermeable to the hydrophobic ethanol-temulawak extract [10].

The biofilm inhibition value for the positive control group is 50% > value determined for the negative control group. This study found that ethanol-temulawak extract inhibited *S. mutans* biofilm formation in all concentrations, and that increased concentration is linearly correlated with increased inhibition value. The correlation test gives a coefficient of correlation of 0.801, indicating a strong, positive relationship between concentration and inhibition value of ethanol-temulawak extract against formation of single-species *S. mutans* biofilm. It is now proved that ethanol-temulawak extract can prevent *S. mutans* biofilm formation, but not as effectively as CHX. The ethanol-temulawak extract

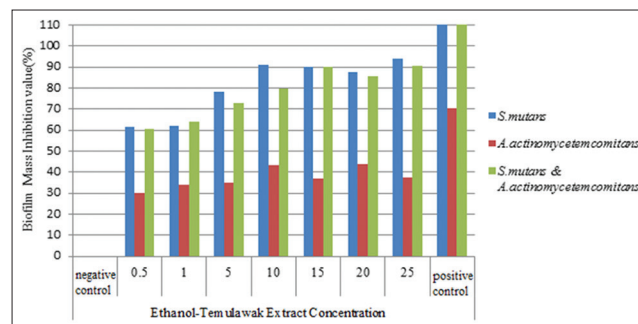


Fig. 1: The Effect of ethanol-temulawak extract on single- and dual-species biofilm formation

works by disrupting cell wall integrity, causing lysis with disruption of the cell wall and membrane, and thus, destroying the ability of *S. mutans* to adhere and form a biofilm [11,4]. The effect of ethanol-temulawak extract against *A. actinomycetemcomitans* biofilm formation was determined in the same way as for *S. mutans*. The study showed that ethanol-temulawak extract could not prevent single-species *A. actinomycetemcomitans* biofilm formation. This resistance to ethanol-temulawak extract is probably caused by the inability of the ethanol-temulawak extract to permeate the cell wall, so that biofilm formation continues [10]. However, the correlation test gave a coefficient of correlation of 0.594, meaning that there was a relationship between the concentration and inhibition value of ethanol-temulawak extract against single-species *A. actinomycetemcomitans* biofilm formation. It was presumed that the ability of ethanol-temulawak extract to inhibit *A. actinomycetemcomitans* biofilm formation is over 25%, but no inhibition value could be determined.

In the dual-species biofilm study, ethanol-temulawak extract could prevent the formation of dual-species biofilm in every concentration. The correlation test gave a coefficient of correlation of 0.920, proving a strong relationship between increased concentration and increased inhibition value. This is probably caused by several interactions between the two bacteria. As a metabolism by product, *S. mutans* produces lactic acid, an important energy source for *A. actinomycetemcomitans*. *S. mutans* was exposed to ethanol-temulawak extract before receiving sucrose, so was already undergoing lysis, no longer producing lactic acid, and thus, diminishing the ability of *A. actinomycetemcomitans* to grow. In turn, the growth of *S. mutans* is probably disturbed by the actinobacilin produced by *A. actinomycetemcomitans*. Thus, in the dual-species biofilm, only *A. actinomycetemcomitans* can survive to form a biofilm; the biofilm formation was probably reduced because the gross number of *A. actinomycetemcomitans* originally introduced was only half the number used in the single-species experiments [12-14].

CONCLUSION

This research concluded that the MIC of ethanol-temulawak extract against *S. mutans* was 5%, while the MBC was 15%. The MIC and MIB of ethanol-temulawak extract against *A. actinomycetemcomitans* could not be determined. *In vitro*, ethanol-temulawak extract could prevent the formation of a single-species biofilm of *S. mutans* and a dual-species biofilm of *S. mutans* and *A. actinomycetemcomitans*. However, ethanol-temulawak extract could not prevent the formation of a single-species

biofilm of *A. actinomycetemcomitans*. The recommendations from this research are to direct a standardization of temulawak extract and to conduct an effectiveness test of ethanol-temulawak extract against biofilm, using non-colorimetric methods (the extract's strong color affects measurements of optical density). Further investigation is also needed into the biomolecular aspects of temulawak resistance in *A. actinomycetemcomitans*.

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