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Research Article

EFFECTIVENESS OF IDENTIFIED JAVANESE TURMERIC ETHANOL EXTRACT FOR THE INHIBITION OF BIOFILM FORMATION BY *STREPTOCOCCUS MUTANS* AND *PORPHYROMONAS GINGIVALIS*

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ABSTRACT

Objective: To study the effectiveness of identified Javanese turmeric ethanol extract (IJTEE) against single and combined biofilm formation by *Streptococcus mutans* and *Porphyromonas gingivalis.*

Methods: *S. mutans* ATCC 25175 and *P. gingivalis* ATCC 33277 were tested for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of IJTEE using microdilution technique. The inhibition of biofilm formation by IJTEE was analyzed using crystal violet assay.

Results: MIC and MBC of IJTEE for *S. mutans* were 5% and 15%, respectively. MIC of IJTEE for the biofilm of *S. mutans* was 1% and for that of *P. gingivalis* was 15%; the MIC of IJTEE for the combined biofilm was 0.5%.

Conclusion: IJTEE was effective in inhibiting single and combined biofilm formation by *S. mutans* and *P. gingivalis*.

Keywords: Biofilm, identified Javanese turmeric ethanol extract, Minimum bactericidal concentration, Minimum inhibitory concentration, *Porphyromonas gingivalis*, *Streptococcus mutans.*

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INTRODUCTION

Indonesia is a nation rich in mega-biodiversity where there are 40,000 endemic plants and 6000 of them are used for herbal medication [1]. Indonesian people use the medicinal plants for self-medication; however, these plants are not prescribed by doctors because no research has been conducted to identify their efficacy; their safety is also unclear [2,3]. *Curcuma xanthorrhiza* Roxb. (Javanese turmeric) is one of the medicinal plants that may be developed as phytopharmaca because some research has identified its effectiveness in relieving some pathological conditions (e.g., liver disease, rheumatic disease, fatigue, and depression). It also has antibacterial, antifungal, antioxidant, and antitumor effects [4]. Xanthorrhizol is known as one of the active compounds in Javanese turmeric and Atsiri oil that has an antibacterial effect against several pathogens in the oral cavity [5].

Bacteria are normal microorganisms found in the oral cavity, but they become pathogenic once they interact with other bacteria within the biofilm. Caries is a disease of the oral cavity and is often caused by *Streptococcus mutans* [6] that form a biofilm. Biofilm formation is initiated by interaction (quorum sensing) between planktonic early colonizing bacteria and the enamel surface of the teeth. After the biofilm is formed, it binds to Gram-negative anaerobic late colonizing bacteria *(Porphyromonas gingivalis),* leading to periodontal disease [7].

A synthetic antibacterial drug, chlorhexidine gluconate (CHx), having a broad spectrum of action against pathogenic bacteria in the oral cavity, has been formulated to prevent caries and periodontal diseases. CHx decreases pellicle formation and inhibits bonding between bacteria and the surface of the teeth. This drug is not yet widely available because it causes many side effects, one of which is biofilm retention [8]. People in Indonesia experience difficulty accessing synthetic drugs because of the lack of effective distribution. Many people use Javanese turmeric to treat diseases. The antibacterial effect of Javanese turmeric is known to result from the component xanthorrhizol, but the composition has not been established. In previous research, xanthorrhizol isolated from Javanese turmeric has been used against planktonic bacteria such as *S. mutans* and *P. gingivalis* [9], but further research is needed to identify their effectiveness in inhibiting biofilm formation by *S. mutans* and *P. gingivalis.* This study aimed to evaluate the effectiveness of identified Javanese turmeric ethanol extract (IJTEE) in inhibiting single or combined biofilm formation by *S. mutans and P. gingivalis*.

METHODS

This study obtained test materials in the form of Javanese turmeric ethanol extract from Balittro, Bogor. The ethanol extract of Javanese turmeric was centrifuged (3000 rpm; 20 min; 37°C), and the top layer was used for identification and later referred as IJTEE. IJTEE was diluted with 10% dimethyl sulfoxide to obtain 0.25%, 0.5%, 1%, 5%, 10%, 15%, 20%, and 25% concentrations of IJTEE solution, according to the dilution formula C1×V1=C2×V2.

Next, 10-µl samples of each bacterium were obtained from the stock of the Oral Biology Laboratory, University of Indonesia, and inserted into brain–heart infusion (BHI) jelly, and incubated in an anaerobic atmosphere, 48 h for *S. mutans* and 72 h for *P. gingivalis.* One resulting colony of each bacterium was inserted into a microcentrifuge tube containing 1 ml phosphate-buffered saline (PBS), and the tube was centrifuged (13,000 rpm; 60 s; 40°C). The supernatant solution was removed from the tube, so that only a pellet layer remained inside the microcentrifuge tube; then, 1 ml of BHI broth was added to the tube, and the contents were homogenized by means of vortex.

Different dilutions of the bacteria–BHI jelly ratio from 10:1 to 10:8 were prepared, and the numbers of bacterial colonies in each dilution were calculated. From the results of the bacterial colony count, a bacteria–BHI jelly ratio of 10:5 was used as the sample because the number of bacterial colonies at this ratio was sufficient.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

In total, 100 µl of each of the 10:5 bacteria–BHI jelly samples was inserted into three types of wells, designated treatment, negative control, and positive control. A 100-µl serial concentration of IJTEE was added to the treatment wells; 100 µl of BHI broth alone was added to the negative control wells; and 100 µl of 0.2% CHx was added to the positive control wells. In addition, a "blank" plate of wells with 100 µl of BHI broth and 100 µl of test material was prepared. Then, the plates were incubated in an anaerobic atmosphere at 37°C for 2 × 24 h for *S. mutans* and 3 × 24 h for *P. gingivalis.* The optical density absorbance value was read by a microplate reader at a wavelength of 450 nm [10].

Test for the effectiveness of identified Javanese turmeric ethanol extract in inhibiting single biofilm

We prepared a BHI broth medium to which 0.2% sucrose was added. Then, each bacterium sample was diluted using the same steps as before until the bacterium–BHI jelly ratio was 10:5.

Well plates labeled with the sample name were prepared. Then, 100-µl suspension of each of the bacteria was added into the treatment wells, the negative control wells, and the positive control wells. Then, the treatment sample was exposed to serial concentrations of IJTEE as high as 100 µl. In the positive control wells, 100 µl of 0.2% CHx was added; in the negative control wells, 100 µl of BHI broth (0.2% sucrose) was added. In addition, a "blank" plate of wells containing 100 µl of BHI broth medium (with 0.2% sucrose) and 100 µl of the test material was prepared. Then, each well plate was incubated in an anaerobic atmosphere at 37°C for 18 h [11].

Test for the effectiveness of identified Javanese turmeric ethanol extract in inhibiting the combined biofilm

BHI broth medium (0.2% sucrose) was prepared with 1 ml vitamin K. Then, the bacteria were diluted using the same steps as before until the bacteria–BHI jelly ratio was 10:5.

Following this, 50 µl of the 10:5 *S. mutans* suspension solution and 50 µl of the 10:5 *P. gingivalis* suspension solution were added to each well. From each treatment well, 100 µl was exposed to various concentrations. In the positive control wells, 100 µl of 0.2% CHx was added; in the negative control wells, 100 µl of BHI broth was added. In addition, a "blank" well plate of wells containing 100 µl of BHI broth and 100 µl of test material was prepared. Each well plate was then incubated in an anaerobic atmosphere at 37°C for 18 h [8].

Crystal violet test

The solution in each well was aspirated, rinsed with 200 µl of PBS solution, fixed for 10 min, and then stained with 0.5% crystal violet solution. To obtain a 0.5% crystal violet solution, a centrifuge tube containing 49.75 ml aquadest was prepared with 0.25 ml of crystal violet solution. Later, 200 µl of crystal violet solution was added into each well, and the plates were incubated at 37°C for 15 min. Then, the solution in each well was rinsed twice with sterile distilled water, 200 µl of 96% ethanol was added, and the plates were incubated at 37°C for 15 min; the plates were inserted into the microplate reader set at 490-nm wavelength to obtain optical density [11,12].

Data results of the inhibition test of biofilm formation were subjected to analysis of variance (ANOVA), bivariate correlation, and linear regression to identify the relationship between the various substances and the amount of biofilm.

RESULTS

The minimum inhibitory concentration (MIC) of IJTEE against *S. mutans* was 5%, and the MBC of IJTEE against *S. mutans* was 15%. However, MIC and MBC against *P. gingivalis* could not be established because no inhibition of *P. gingivalis* exceeded 90% (Table 1).

Fig. 1 shows the effectiveness of IJTEE in inhibiting the formation of *S. mutans* biofilms starting at a concentration of 1%, so that the $MBEC_{50}$ of *S. mutans* was set at a concentration of 1%. One-way ANOVA showed significant differences ($p<0.05$) between the treatment samples and the negative and positive control samples. The bivariate correlation test between IJTEE concentration and biofilm inhibition percentage showed an increase in IJTEE concentration with increased biofilm inhibition ($p<0.025$). The linear regression test showed the closeness of the relationship ($R^2=0.79$) between biofilm inhibition percentage and IJTEE concentration. 79% inhibition of biofilm formation was estimated to be caused by IJTEE, whereas 21% inhibition of biofilm formation was caused by other factors (the number of bacteria that could not be ascertained in each treatment sample). The regression formula obtained was as follows:

$$Y = 48.75 + 2.41(x)$$

This proved the existence of linear regression between IJTEE and inhibition of biofilm formation. With every change in concentration, the inhibition of biofilm increased by 2.416%.

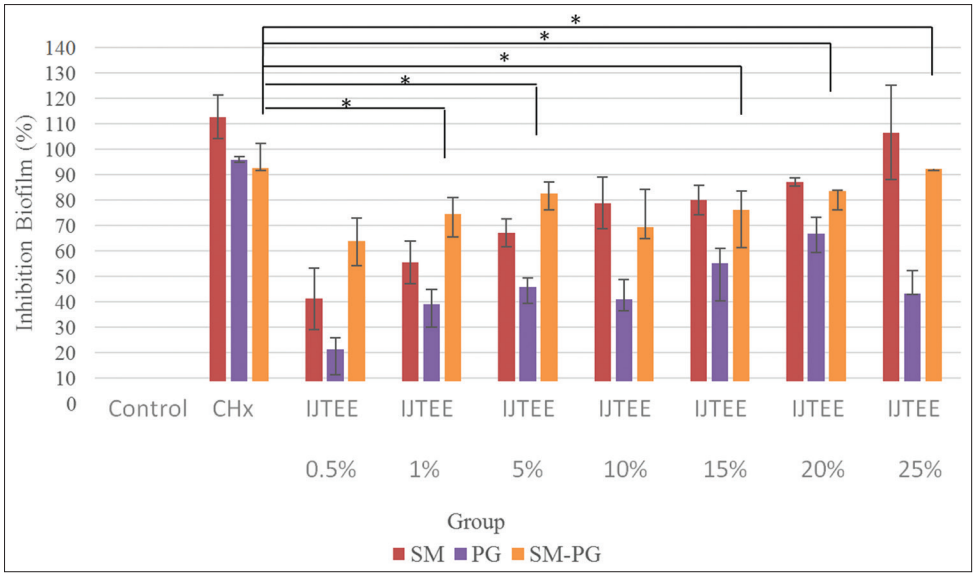

Fig. 1: Effectiveness of IJTEE in inhibiting biofilm formation. Abbreviations: IJTEE: Identified Javanese turmeric ethanol extract, CHx: Chlorhexidine gluconate, PG: Porphyromonas gingivalis, SM: *Streptococcus mutans*

Table 1: The percentage inhibition of *Streptococcus mutans* and *Porphyromonas gingivalis* by different concentrations of identified Javanese turmeric ethanol extract

Sample	Inhibition (%)±SD	
	*Streptococcus mutans**	*Porphyromonas gingivalis**
Negative control	0	0
Positive control	100.19±0.94	101.88±2.17
IJTEE 0.25%	85.84±1.52	65.02±6.25
IJTEE 0.5%	86.74±1.45	81.38±2.33
IJTEE 1%	89.42±2.21	71.71±9.79
IJTEE 5%	92.85±1.00 (MIC)	75.72±2.95
IJTEE 10%	95.17±2.59	84.46±3.48
IJTEE 15%	100.48±0.67 (MBC)	79.8±2.57
IJTEE 20%	100.57±0.20	79.32±3.61
IJTEE 25%	100.23±0.02	82.25±2.46
10% DMSO solution	16.51±2.14	13.41±3.93

**n*=2. DMSO: Dimethyl sulfoxide, IJTEE: Identified Javanese turmeric ethanol extract, MBC: Minimum bactericidal concentration, MIC: Minimum inhibitory concentration, SD: Standard deviation

The effectiveness of IJTEE in inhibiting the formation of biofilm by *P. gingivalis* started at a concentration of 15%, so that $MBEC_{50}$ of *P. gingivalis* was defined at the concentration of 15%. One-way ANOVA showed significant differences ($p<0.05$) between treatment samples and negative and positive control samples. The bivariate correlation test between IJTEE concentration and biofilm inhibition percentage showed an increase in IJTEE concentration with increased biofilm inhibition ($p<0.025$). The linear regression test showed the closeness of the relationship ($R^2=0.36$) between biofilm inhibition percentage and IJTEE concentration. In addition, it is indicated that 36% inhibition of biofilm formation was caused by IJTEE, and 64% inhibition of biofilm formation was caused by other factors (the formation of older [24-h] *P. gingivalis* biofilms). The regression formula obtained was as follows:

$$Y = 33.95 + 0.96(x)$$

This formula shows the closeness of the relationship between IJTEE and inhibition of biofilm formation. With every change in concentration, the inhibition of biofilm increased by 0.96%.

The effectiveness of IJTEE in inhibiting the formation of combined biofilm by *S. mutans* and *P. gingivalis* was started at a concentration of 0.5%, so that the $MBEC_{50}$ of the combination of *S. mutans* and *P gingivalis* was at the concentration of 0.5%. One-way ANOVA showed a significant difference ($p<0.05$) between the treatment samples and negative control samples, but not between the treatment samples and positive control samples ($p>0.05$). The bivariate correlation test between IJTEE concentration and biofilm inhibition percentage showed an increase in IJTEE concentration with increased biofilm inhibition ($p<0.025$). The linear regression test showed the closeness of the relationship ($R^2=0.34$) between biofilm inhibition percentage and IJTEE concentration. In the result of bivariate correlation test between IJTEE concentration and inhibition of biofilm formation, correlation coefficient value was 0.58, indicating a positive correlation. In addition, R^2 (0.34) indicated that 34% inhibition of biofilm formation was caused by IJTEE, and 66% inhibition of biofilm formation was caused by other factors. The regression formula obtained was as follows:

$$Y = 69.25 + 0.74(x)$$

This formula showed the closeness of the relationship between IJTEE and inhibition of biofilm formation. With every change in concentration, the inhibition of biofilm increased by 0.74%.

DISCUSSION

IJTEE has different antibacterial activities against *S. mutans* and *P. gingivalis,* presumably because of differences in cell wall. *S. mutans* have thick cell walls consisting of peptidoglycan (50–90%), whereas *P. gingivalis* has cell walls composed of a thin layer of peptidoglycan and an additional outer membrane composed of lipids, with a periplasmic space between. In addition, the composition of the bacterial wall of *P. gingivalis* is more complex because lipopolysaccharide senses before a foreign object penetrates the cell, and hydrophobic porin lipoprotein then limits the diffusion of foreign matter into the cell. Further research is needed to determine the MIC and MBC of IJTEE against *P. gingivalis.* Our findings can also be compared with those of previous research in which MIC and MBC of IJTEE against *S. mutans* were identified [13]. Determinations of MIC may differ as a result of the difference in extracts used due to the selection of raw materials that cause the xanthorrhizol levels to be different. The MIC and MBC values of *P. gingivalis* that cannot be determined may be similar to those of previous studies although the solvents used in this study are different [14].

The effectiveness of identified Javanese turmeric ethanol extract in inhibiting the biofilm formation by *S. mutans*

ANOVA showed significant differences ($p<0.05$) between treatment samples and negative and positive control samples. We suspected that IJTEE had an effect on inhibiting the formation of biofilm by *S. mutans*, but its effectiveness was not equivalent to that of CHx. This suspicion is supported by previous research, in which CHx had strong antibacterial effects in penetrating biofilm extracellular matrix. In addition, *S. mutans* has a simple peptidoglycan cell wall, which can be easily penetrated by antibacterial agents [8].

The effectiveness of identified Javanese turmeric ethanol extract in inhibiting biofilm formation by *Porphyromonas gingivalis*

Fig. 1 shows that, at IJTEE concentrations of 15% and 20%, the formation of biofilm by *P. gingivalis* was inhibited; therefore, we can conclude that there was antibacterial activity. It may be caused by xanthorrhizol and flavonoids contained in IJTEE that have antibacterial effects. Meanwhile, at IJTEE concentrations of 15% and 20%, the degree of inhibition of *P. gingivalis* biofilm formation did not reach more than 90%. This finding and results of previous studies indicate that antibacterial agents inhibit the formation of biofilms at lower MICs, and antibacterial activity of xanthorrhizol inhibits the formation of biofilm by *P. gingivalis* at higher concentrations [5].

ANOVA showed significant differences ($p<0.05$) between the treatment samples and negative and positive control samples. We suspect that IJTEE has an effect on inhibiting the formation of biofilm by *P. gingivalis*, but its effectiveness was not equivalent to that of CHx, which has a strong antibacterial effect in penetrating the biofilm extracellular matrix. In addition, *P. gingivalis* has a more complex cell wall that can limit the diffusion of foreign matter into cells [13].

The effectiveness of identified Javanese turmeric ethanol extract in inhibiting combined biofilm formation by *Streptococcus mutans* and *Porphyromonas gingivalis*

Fig. 1 shows the inhibition of the formation of combined biofilm by *S. mutans* and *P. gingivalis* by IJTEE occurred at concentrations of 0.5% and increased with increasing IJTEE concentrations. This effect may have been caused by xanthorrhizol and flavonoids contained in IJTEE that have antibacterial effects. This is supported by previous research which stated that IJTEE was able to decrease biofilm viability [13].

The ANOVA test between the treatment sample and the negative control sample showed a significant difference ($p<0.05$), and the positive control sample showed no significant difference ($p>0.05$). It is suspected that IJTEE had an effect on inhibiting the formation of combined biofilm by *S. mutans* and *P. gingivalis*, and the effectiveness of IJTEE was equivalent to CHx. The interactions between bacteria may play a role in inhibiting biofilm formation [15]. It is presumed that quorum sensing between bacteria causes antibacterial agents to be more effective. This is supported by previous research, wherein *P. gingivalis* inhibited quorum sensing among *S. mutans,* and therefore, *S. mutans* failed to interact [15].

CONCLUSION

IJTEE effectively inhibited the formation of biofilm by *S. mutans* starting at a concentration of 1% and the formation of biofilm by *P. gingivalis* starting at a concentration of 15%; it inhibited the combined biofilm formation by *S. mutans* and *P. gingivalis* starting at a concentration of 0.5%.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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