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Research Article

EFFECTIVENESS OF A JAVANESE TURMERIC ETHANOL EXTRACT FOR ERADICATING *STREPTOCOCCUS MUTANS* AND *PORPHYROMONAS GINGIVALIS* BIOFILMS

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ABSTRACT

Objective: We aimed to determine the effectiveness of an identified Javanese turmeric ethanolic extract (IIJTEE) for eradicating biofilms formed by *Streptococcus mutans*, *Porphyromonas gingivalis*, or both.

Methods: Such biofilms during different growth phases were exposed to various concentrations of an IJTEE to determine its effects on bacterial proliferation.

Results: The effectiveness of the IJTEE in eradicating the S. mutans biofilm was concentration-dependent but not when used to treat *P. gingivalis* and *S. mutans* - *P. gingivalis* biofilms.

Conclusion: The effectiveness of the IJTEE for eradicating biofilms formed by *S. mutans*, *P. gingivalis,* and *S. mutans* plus *P. gingivalis* biofilms depended on the growth phase of the biofilm. Thus, IJTEE eradicated biofilms formed by *S. mutans*, *P. gingivalis*, or both.

Keywords: Biofilm eradication, Dual-species biofilm, Identified Javanese turmeric ethanolic extract, Single-species biofilm, *Streptococcus mutans, Porphyromonas gingivalis.*

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INTRODUCTION

Indonesian Basic Health Survey conducted in 2013 reveals that the percentage of the population with dental and oral problems (25.9%) increased by 2.7% in 2007 [1]. The most prevalent oral health problems include caries and periodontal disease, which are initiated by biofilm formation [2-4]. Biofilms comprise bacterial microcolonies that adhere to tooth surfaces. An extracellular matrix protects the bacteria from antibiotics and hosts defense mechanisms. Bacteria in biofilms undergo multiple phenotypic changes, mediated by differential gene expression, which confer increased resistance to antimicrobial agents in contrast to their planktonic forms [4,5]. *Streptococcus mutans* and *Porphyromonas gingivalis* form biofilms, individually, or together, which cause diseases of the oral cavity [3,4].

S. mutans in biofilms produce products such as lactic acid through the fermentation of carbohydrates, which decrease the pH of the oral cavity and initiates demineralization causing Ca^{2+} and PO_4^{2-} to elute from teeth [3]. *P. gingivalis* forms biofilms on the surface of the gingival sulcus. Virulence factors expressed by *P. gingivalis* can cause inflammation of periodontal tissues, degrade the intercellular junction, and activate osteoclasts that cause alveolar bone resorption [6].

Chlorhexidine gluconate is a broad-spectrum bisbiguanide that is considered the gold standard for curing oral health problems caused by pathogenic bacteria. However, long-term administration of this compound can change the composition of the microbiota in the oral cavity. Notably, the distribution of chlorhexidine gluconate in remote areas in Indonesia is limited [7]. Alternative antibacterial agents from plants with effects similar to those of chlorhexidine gluconate are needed so they can be easily obtained by the community.

Javanese turmeric (*Curcuma xanthorrhiza* Roxb.), particularly its rhizomes, is one of nine superior medicinal plants characterized by Indonesia's National Agency of Drug and Food Control. Javanese turmeric is the third most frequently used plant by the Indonesian community to resolve public health problems [8,9]. Javanese turmeric is active against intraoral bacteria such as *S. mutans* and *P. gingivalis* as shown by the studies of Hwang *et al*. [10] who tested an xanthorrhizol isolate. Moreover, the inhibitory activity of xanthorrhizol against *S. mutants* was demonstrated by Rukayadi and Hwang [10,11].

Extracts of medicinal plants extracts are most frequently used to test their effects *in vitro*, and the characterization of an extract's components is an absolute requirement to implement public health and clinical measures to prevent and treat oral diseases. Javanese turmeric ethanolic extract (IJTEE) is an antibacterial agent. However, its active components and organoleptic properties are unknown. Moreover, research on the effects of IJTEEs against biofilms remains to be determined. To address these gaps in our knowledge, we conducted a study to determine whether the effects of a IJTEE on biofilms formed by *S. mutans*, *P. gingivalis,* or both, are sufficient for use in personal and clinical oral hygiene.

METHODS

IJTEE was obtained from Balai Penelitian Tanaman Rempah dan Obat, Bogor, which was prepared using the maceration method. The extract was centrifuged at 3000 rpm for 20 min, which fractionated the extract into four layers with different consistencies. The top layer was taken and subjected to phytochemical tests, and the content of xanthorrhizol was measured.

S. mutans ATCC 27175 serotype C and *P gingivalis* (ATCC 33277) were inoculated into brain-heart infusion (BHI) medium or BHI agar supplemented with Vitamin K for culturing *P. gingivalis*. The two species were inoculated into a jar containing at anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% O_2 and incubated at 37°C for 48 h or 72 h for *S. mutans* or *P. gingivalis*, respectively. After incubation, *S. mutans* and *P. gingivalis* suspensions were prepared by inserting one portion of a colony into 1 ml of phosphate-buffered saline and centrifuged at 13,000 rpm for 60 s. The supernatant was aspirated, and 1 ml of BHI broth supplemented with Vitamin K added to the bacterial pellet.

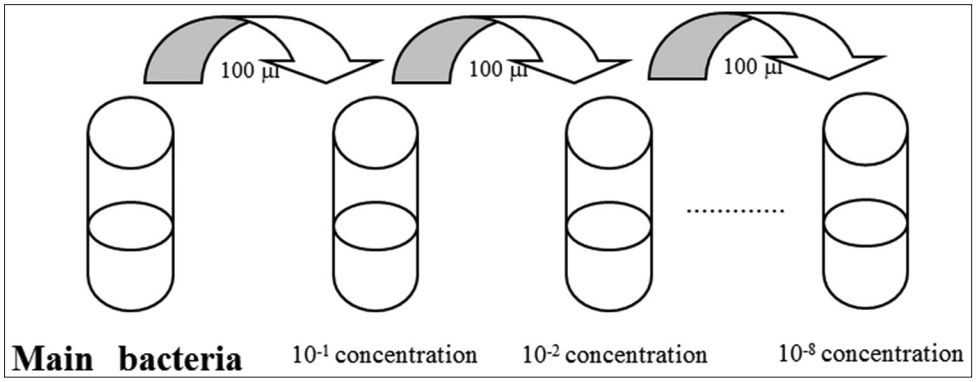


Fig. 1: Serial dilution of bacterial suspensions

The cell counts of the suspensions of *S. mutans* and *P. gingivalis* were determined using serial 10-fold dilutions of the stock bacterial suspensions (Fig. 1). The diluted suspensions were inoculated onto BHI agar or BHI agar with Vitamin K and added to a jar containing an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% O_2 and incubated at 37°C for 48 h or 72 h for *S. mutans* or *P. gingivalis*, respectively. After incubation, colony counts were determined.

Biofilms were formed using a BHI broth biofilm model containing 0.2% sucrose or BHI broth with Vitamin K and 0.2% sucrose solution for *P. gingivalis*. A 96-well plate was equally divided into a treatment group containing bacteria and medium and a blank group containing the only medium. Biofilms were formed from each species by adding 100 µl of BHI broth with 0.2% sucrose or BHI broth with Vitamin K and 0.2% sucrose containing *S. mutans* or *P. gingivalis*, which was added to the treatment well, and 100 µl of BHI broth with 0.2% sucrose or BHI broth with Vitamin K was added to the blank well. The 96-well plate was then inserted into a jar containing an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% O_2, which was incubated at 37°C for 4 h, 12 h, and 24 h.

Biofilms comprising both bacterial species (dual-species biofilms) were formed by adding 50 µl of BHI broth with Vitamin K and 0.2% sucrose containing *S. mutans* and 50 µl of BHI broth with Vitamin K and 0.2% sucrose containing *P. gingivalis* into the treatment well. The blank well contained 100 µl of BHI broth with Vitamin K. The 96-well plate was then inserted into a jar containing an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% O_2, which was incubated at 37°C for 4 h, 12 h, and 24 h. The supernatants were aspirated without touching the base of the plates and were then rinsed once to eliminate planktonic bacteria. Single- and dual-species biofilms were treated with 0.5%, 1%, and 5% solutions of the IJTEE, 0.2% *chlorhexidine gluconate* (positive control), and BHI broth with 0.2% sucrose or BHI broth with Vitamin K, and 0.2% sucrose (negative control). The 96-well plate was then inserted into a jar containing an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% O_2 and incubated at 37°C for 60 min. These procedures were performed in triplicate.

The supernatant was aspirated without touching the base of the plate, and 5 mg/ml of MTT was then added to each treatment or blank well. The 96-well plate was then inserted into a jar containing an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% O_2, and incubated at 37 °C for 3 h. *Acidified isopropanol* (100 µl) was added to each well, and the 96-well plate was placed on an orbital shaker (80 rpm) for 60 min. Optical density (490 nm) values were determined using a microplate reader.

RESULTS

The percentage eradication of biofilms formed by *S. mutans, P. gingivalis,* or both was determined as follows:

The $MBEC_{90}$ value of the IJTEE for the adhesion phase of the biofilm was 0.5%. There was a significant difference between 0.5% and 25% IJTEE groups and the negative control. The activity of the IJTEE comparable to that of the positive control was observed only at a concentration of 20%. The $MBEC_{50}$ and $MBEC_{90}$ values of the IJTEE for an *S. mutans* biofilm during the accumulation phase were 0.5% and 1%, respectively. There was a significant difference between 0.5% and 25% IJTEE groups and the negative control. The activity of the IJTEE comparable to that of the positive control was observed only at concentrations of 15% and 20%. The $MBEC_{50}$ and $MBEC_{90}$ values of the IJTEE for the *S. mutans* biofilm during the maturation phase were 0.5% and 15%, respectively, and differed significantly between the 0.5% and 25% IJTEE groups and the negative control, while the activity of the IJTEE was comparable with the positive control only at concentrations of 20% and 25%.

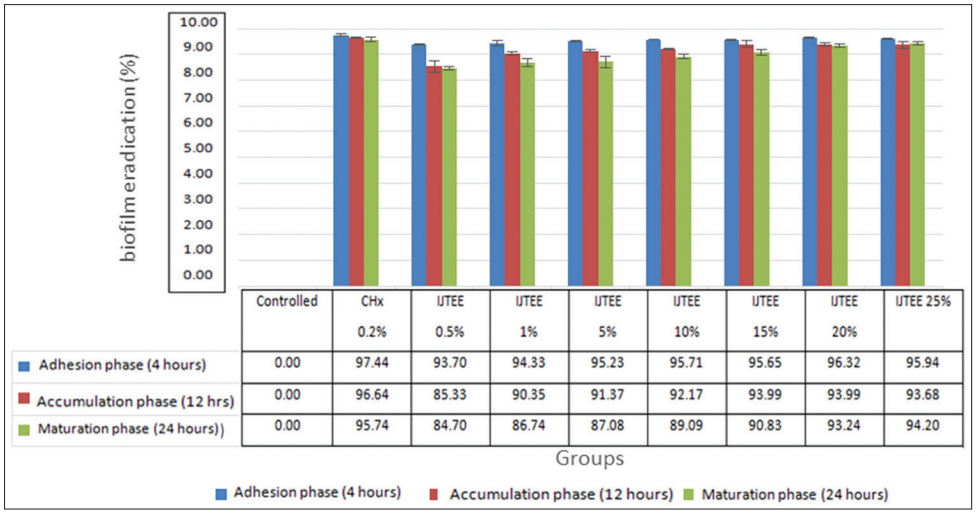
Fig. 2 shows an increase in adhesion of *S. mutans* to the biofilm as well as in its accumulation and an increase in the percentage eradication during the maturation phase. There was a statistically significant effect of the IJTEE on the percentage eradication of the *S. mutans* biofilm during all growth phases. Further, there was a significant decrease in the percentage eradication of the *S. mutans* biofilm with time. There was a significant association between treatment with the IJTEE and the inhibition of biofilm eradication with time.

The $MBEC_{90}$ values of the IJTEE for the adhesion and accumulation phases of the *P. gingivalis* biofilm were 0.5% and 10%, respectively. There were significant differences between 0.5% and 25% IJTEE groups and the negative control, and comparable effectiveness to the positive control was observed only for the 1% and 10% concentrations. The $MBEC_{90}$ value for the adhesion phase of a *P. gingivalis* biofilm was 0.5%. There were significant differences between 0.5% and 25% IJTEE groups and the negative control, although the effectiveness was not comparable with that of the positive control. The $MBEC_{50}$ value of the IJTEE on the maturation phase of the *P gingivalis* biofilm was 10%. There were significant differences between 0.5% and 25% IJTEE groups and negative control, although there was no comparable effectiveness compared with the positive control.

Fig. 2 shows that there were increases in the eradication of the biofilm formed by *S. mutans* during the adhesion, accumulation, and maturation phases as a function of increasing concentrations of the IJTEE. There was a significant association between treatment with the IJTEE and the percentage eradication of the *S. mutans* biofilm in all phases of growth as well as with the age of the biofilm.

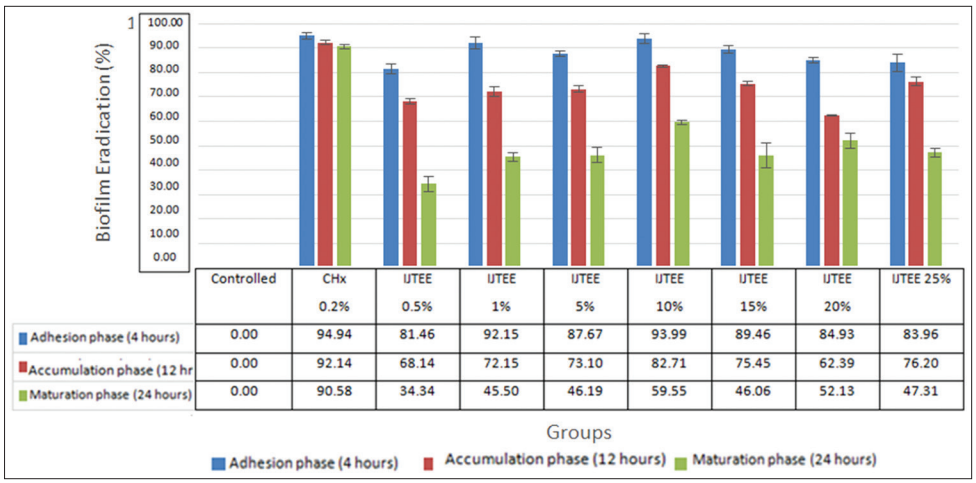
Fig. 3 shows inconsistency in the percentage eradication during the adhesion, accumulation, and maturation phases of the biofilm formed by *P. gingivalis* as a function of increasing concentrations of the IJTEE. There were no significant differences in the effects of the IJTEE on the percentage eradication of the biofilm as a function of growth phase. Further, the percentage eradication of the *P. gingivalis* biofilm did not increase as a function of time.

The $MBEC_{90}$ of the IJTEE for *S. mutans* and *P. gingivalis* during the adhesion phase of biofilm formation was 0.5% concentration, and



	Controlled	CHx 0.2%	IJTEE 0.5%	IJTEE 1%	IJTEE 5%	IJTEE 10%	IJTEE 15%	IJTEE 20%	IJTEE 25%
Adhesion phase (4 hours)	0.00	97.44	93.70	94.33	95.23	95.71	95.65	96.32	95.94
Accumulation phase (12 hrs)	0.00	96.64	85.33	90.35	91.37	92.17	93.99	93.99	93.68
Maturation phase (24 hours))	0.00	95.74	84.70	86.74	87.08	89.09	90.83	93.24	94.20

Fig. 2: Eradication of an *Streptococcus mutans* biofilm using a Javanese turmeric ethanol extract



	Controlled	CHx 0.2%	IJTEE 0.5%	IJTEE 1%	IJTEE 5%	IJTEE 10%	IJTEE 15%	IJTEE 20%	IJTEE 25%
Adhesion phase (4 hours)	0.00	94.94	81.46	92.15	87.67	93.99	89.46	84.93	83.96
Accumulation phase (12 hr	0.00	92.14	68.14	72.15	73.10	82.71	75.45	62.39	76.20
Maturation phase (24 hours)	0.00	90.58	34.34	45.50	46.19	59.55	46.06	52.13	47.31

Fig. 3: Javanese turmeric ethanolic extract eradication of *Porphyromonas gingivalis* biofilms

the $MBEC_{90}$ for the accumulation phase for *P. gingivalis* was presented 1%. There were significant differences between 0.5% and 25% IJTEE groups and the negative control, but no comparable effectiveness compared with the positive control. The $MBEC_{50}$ of the IJTEE for *S. mutans* and *P. gingivalis* during the accumulation phase was 0.5%. There was a significant difference between 0.5% and 25% IJTEE groups and negative control, although there was no comparable effectiveness compared with the positive control. The $MBEC_{50}$ of the IJTEE on *S. mutans* and *P. gingivalis* during the maturation phase of biofilm formation was 10%. There were significant differences between 0.5% and 25% IJTEE groups and the negative control, although the influence on effectiveness was not comparable with that of the positive control.

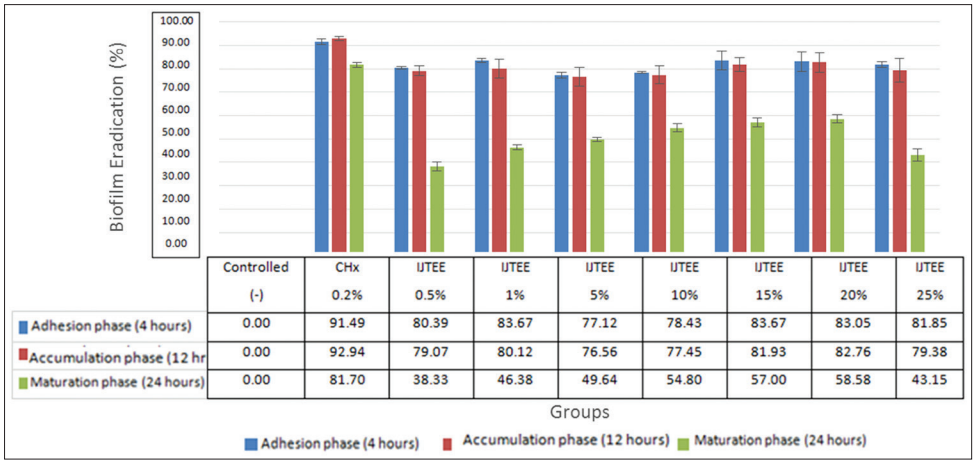
Fig. 4 shows inconsistent percentage eradication during the adhesion and accumulation phases of biofilm formation by *S. mutans* and *P. gingivalis*. In contrast, during the maturation phase, the percentage of biofilm eradication increased. There was no significant association between treatments with the IJTEE on the percentage eradication of the dual-species biofilm during all phases of growth. Further, the percentage eradication of the dual biofilm decreased as a function of time. Statistical analysis showed a significant association between an increase in the age of the biofilm with a decrease in the eradication of the biofilm formed by *P. gingivalis*.

DISCUSSION

The $MBEC_{50}$ of the IJTEE was 0.5% for the accumulation and maturation phases of the *S. mutans* biofilm, and the $MBEC_{90}$ during the adhesion phase was 0.5%. The effects of THE IJTEE on all treatment groups of *S. mutans* biofilms during all growth phases differed significantly from those of the negative control as well as on the adhesion, accumulation, and maturation phases. The effectiveness for the eradication of biofilms of the IJTEE was comparable to that of 0.2% *chlorhexidine gluconate* and was concentration-dependent. IJTEE inhibits the viability of *S. mutans* in a concentration-dependent manner. Further, Rukayadi and Hwang found that the effectiveness of an IJTEE for eradicating *S. mutans* biofilms depends on its concentration, the exposure time as well as on the growth phase of the biofilm.

The ability of the IJTEE to eradicate an *S. mutans* biofilm is conferred by the phenolic hydroxyl group of xanthorrhizol, which forms a covalent bond with molecules in the bacterium's cell membrane. S. *mutans* has a relatively simple cellular membrane comprising thick layers of peptidoglycans bound to *teichoic acid*, which facilitates the penetration of the cell by phenolic compounds. The binding of a hydroxyl group to the cell membrane alters its permeability and is mediated by peptidoglycans, leading to the disruption of the cell membrane and subsequent cell lysis [12-14].

The $MBEC_{50}$ of the IJTEE for the adhesion and accumulation phases of biofilm formation by *P. gingivalis* was 0.5% and 10% for the maturation phase. The $MBEC_{90}$ for the adhesion phase was 1%. The percentage eradication of biofilm formation by the treatment groups during the growth phase of *P. gingivalis* biofilm formation differed significantly compared with the negative control. The effectiveness of the IJTEE



	Controlled (-)	CHx 0.2%	IJTEE 0.5%	IJTEE 1%	IJTEE 5%	IJTEE 10%	IJTEE 15%	IJTEE 20%	IJTEE 25%
Adhesion phase (4 hours)	0.00	91.49	80.39	83.67	77.12	78.43	83.67	83.05	81.85
Accumulation phase (12 hr	0.00	92.94	79.07	80.12	76.56	77.45	81.93	82.76	79.38
Maturation phase (24 hours)	0.00	81.70	38.33	46.38	49.64	54.80	57.00	58.58	43.15

Fig. 4: Effectiveness of the Javanese turmeric ethanolic extract in biofilms formed by *Streptococcus mutans* combined with *P. gingivalis*

was comparable to that of 0.2% *chlorhexidine gluconate*. There was no significant dependence on the IJTEE concentration and its ability to eradicate the *P. gingivalis* biofilm.

The effectiveness of the IJTEE for eradicating the *P. gingivalis* biofilm during the accumulation and maturation phases was lower compared with that for the dual-species biofilm. This may be explained by factors such as the higher resistance of the *P. gingivalis* biofilm as well as differences in the structures of the membranes of the two bacterial species. The cell membrane of *P. gingivalis* is more complex compared to that of *S. mutans* because the former comprises outer and inner (cytoplasmic membrane) membranes. The thin peptidoglycan layer rests under the outer membrane, separated by the periplasmic space. The periplasmic space contains peptidoglycans as well as enzymes and other small and large molecules.

The outer surface of the membrane of a Gram-negative bacterium consists of a lipopolysaccharide composed of A-lipid and O-polysaccharide moieties. The A-lipid (endotoxin) is toxic to humans and animals. The antigenic properties of the O-polysaccharide antigen differ among bacterial species and subspecies. The lipid imparts impermeability to the cell membrane that prevents molecules from diffusing into the cell. During the accumulation and maturation phases of biofilm formation, extracellular polymeric substances (EPS) begin to form. The EPS acts as a barrier against antibiotics, bacteria, and the components of host defense systems. These cause the *P. gingivalis* biofilm to be more difficult to penetrate by the IJTEE [12-14].

The $MBEC_{50}$ of the IJTEE for both the adhesion and accumulation phases of the dual-species biofilm was 0.5% and 10% for the maturation phase. The $MBEC_{90}$ for the adhesion phase of the dual-species biofilm was 1%. There were significant differences between the negative and positive controls among the IJTEE treatment groups of the dual-species biofilm during all growth phases. There was no significant concentration-dependence on the effectiveness of the IJTEE. The effectiveness of the IJTEE for eradicating the dual-species biofilm was not comparable with that of the positive control. Moreover, there was no significant association between the concentration of the IJTEE and the effectiveness of the eradication of the dual-species biofilm during all phases of growth. The effectiveness of the IJTEE for eradicating the dual-species biofilm was intermediate compared with that of the biofilm formed by the individual species. This may be explained by the interactions between *S. mutans* and *P. gingivalis* in the biofilm. For example, *P. gingivalis* may affect the activity of the quorum sensing system of *S. mutans* by inactivating its competence stimulating peptide. Moreover, *P. gingivalis* can be destroyed by bacteriocins produced by *S. mutans* in a nutrient-limited environment [15].

The percentage eradication by the IJTEE decreased with time for the individual and dual-species biofilms. These findings are consistent with those of Rukayadi and Hwang who found that xanthorrhizol eradicated the biofilm formed by *S. mutants* during the adhesion phase, *and* xanthorrhizol eradicated 76% of the biofilm formed by *S. mutans* during the maturation phase.

Bacteria are most sensitive to antimicrobial agents and the host's immune response during the maturation phase of biofilm formation. During the maturation phase, bacteria grow slowly and use most of their energy to produce EPS, which prevent antibacterial agents from penetrating the cell membrane. When antibacterial agents penetrate the biofilm, they may be inactivated by the EPS, degradative enzymes, other antibacterial, or all of these agents. The change in pH in a biofilm caused by the accumulation of acid produced by bacterial metabolism can affect the activities of antimicrobials. Further, bacteria that compose a biofilm communicate through quorum sensing, which helps to coordinate the metabolic activities of the bacterial populations and acts as an adaptation medium against a hostile environment. Moreover, bacteria in a biofilm freely engage in horizontal gene transfer that may increase bacterial virulence and the resistance of a biofilm to inhibitors. Bacteria may enter a quiescent state when nutrients are limited, which protects them from cytotoxic agents [14-17].

CONCLUSION

The IJTEE was effective in eradicating biofilms formed individually or together by two important bacterial pathogens at concentrations that are compatible with application to routine oral hygiene and in the clinic for preventing and treating biofilm-associated diseases.

CONFLICTS OF INTEREST

The author reports no conflicts of interest.

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