ORIGINAL ARTICLE

Antibiofilm effect of C-10 massoia lactone toward polymicrobial oral biofilms

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ABSTRACT

This study is aimed to test the efficacy of C-10 Massoia lactone in oral polymicrobial degradation. Polymicrobial of *Streptococcus sanguinis, Streptococcus mutans, Lactobacillus acidophilus,* and *Actinomyces viscosus* were studied. C-10 Massoia lactone against biofilm degradation was investigated using modified crystal violet for biofilm staining. The effectiveness of C-10 Massoia lactone against biofilms was calculated by the minimum biofilm inhibitory concentration ($MBIC_{50}$) and the minimum value of biofilm eradication concentration ($MBEC_{50}$). Scanning electron microscope was used to study biofilm cell viability and morphological changes. The results showed a degradation effect of C-10 Massoia lactone against mature oral polymicrobial at 0.25% v/v. C-10 Massoia lactone can degrade polymicrobial biofilms of *S. mutans, S. sanguinis*, *L. acidophilus* and *A. viscosus*. This compound can destroy the extracellular polymeric substances (EPS) of polymicrobial biofilms. The potential application of C-10 Massoia lactone for anti-polymicrobial medication should be applied in such a way that any negative effects are minimized. Further research is needed to confirm the findings of this study.

Key words: *Actinomyces viscosus*, C10 Massoia lactone, *Lactobacillus acidophilus*, polymicrobial biofilms, scanning electron microscope, *Streptococcus* spp

INTRODUCTION

Biofilms show high tolerance to antibiotics.[1] Biofilms in the oral cavity, known as dental plaque, are formed by many microorganisms adhering to the extracellular polymeric substances (EPS),[2] resulting in gingivitis, periodontitis, or periimplantitis.[3]

Previously, most research on biofilm eradication are directed to single microbial species. However, there are interactions among microorganisms in polymicrobial, which contribute to bacterial survival and virulence.[4] Synergism interactions among polymicrobial microorganisms can modify the environmental condition of living biofilms and can increase their resistance to antimicrobials.[5]

Mouthwash can prevent dental caries. Most mouthwash contains an active substance such as chlorhexidine, cetylpyridinium chloride, fluoride and has been studied to be effective against dental plaque. However, the long-term use of those active substances includes irritation of the digestive tract and discoloration of the teeth.[6]

In the present work, we have investigated the efficacy of C-10 Massoia lactone towards oral polymicrobial cultures consisting of four different oral bacterial species,

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Streptococcus sanguinis, Streptococcus mutans, Lactobacillus acidophilus and *Actinomyces viscosus*. Because those bacteria can cause tooth decay, our study could provide new possibilities to improve oral health.

MATERIALS AND METHODS

Planktonic minimum inhibitory concentration assay

Cultures of *S. sanguinis, S. mutans, L. acidophilus,* and *A. viscosus* were grown overnight in brain heart infusion (BHI) broth medium at 37°C then diluted to an inoculum of approximately 1 × 10^8 CFU/ml. Inhibitory concentrations of the test compound were evaluated by microdilution methods using a 96-well polystyrene flat bottom. As much as 100 µL of final solution containing C-10 Massoia lactone with different concentrations (1% v/v; 0.5% v/v; 0.25% v/v; 0.125% v/v), medium and microbe suspension, were added to each well. Diluting control using a medium with 1% dimethyl sulfoxide (DMSO), and negative control using a microbe suspension. Positive control using a microbe suspension with listerin 1% v/v and medium control using a medium without microbe growth. All tests were conducted in triplicate. Plates were incubated at 37°C for 24 h for the intermediate phase biofilm and 48 h for the maturate phase biofilm, and the optical density reading was conducted with a microplate reader at the wavelength of 595 nm. The percentages of inhibition and degradation of the replicate tests using optical density (OD) were used to determine MIC_{50}. Cut off point was determined by the formula: MIC_{50}= (OD control-OD blank) × 50/100. The OD value was near to cut off point, which is the MIC_{50} value.[7] When the inhibition effect of concentration was more than or equal to 50%, this determined the MIC_{50} value.

In vitro biofilm formation inhibition assay

The effect of C-10 massoia lactone towards biofilm formation was conducted in 96-well polystyrene flat-bottom microtiter plate[7] in an anaerobic condition (5% CO_2). The purpose of the anaerobic condition was to increase the formation of the biofilm.

Serial concentrations of C-10 Massoia lactone were used. The positive control used mouthwash Listerine® 1% v/v, and media control used DMSO 1% v/v. BHI containing 2% b/v sucrose, bacterial suspension individually for mono-species and four bacteria (*S. sanguinis, S. mutans, L. acidophilus* and *A. viscosus*) for polymicrobial and test compounds in various concentrations were added to each well. After the incubation at 37°C for 24 h and 48 h under anaerobic condition, the culture medium was removed and rinsed with sterile aquadest. A 125 µL solution of crystal violet 1% (v/v) was added to each well to stain the biofilm. Plates were incubated in room temperature for 15 min. After the incubation process, the biofilm was washed with tap water.[8] Next, 200 µL ethanol 96% were added to each well. Reading is done 595 nm. The amount of sample that could inhibit at least 50% of the biofilm formation was considered as $MBIC_{50}$.[9,10]

In vitro biofilm degradation assay

The efficacy of C-10 Massoia lactone on established oral polymicrobial consisting of *S. sanguinis, S. mutans, L. acidophilus*, and *A. viscosus* was studied with the incubation process in an anaerobic condition.[7] Biofilms were inserted into each microtiter plate and incubated at 37°C for 24 h and 48 h in an anaerobic condition (5% CO_2). After the incubation, plates were washed and 100 µL of media contained C-10 Massoia lactone was added to each washed-well. Plates were again incubated for 24 h to form the intermediate phase biofilm and 48 h to form the maturate biofilm.

Scanning electron microscope analysis

For scanning electron microscope (SEM) analysis, mono-species and polymicrobial were grown on the coverslip in the presence of various concentrations of C-10 Massoia lactone for 24 h at 37°C under an anaerobic condition. Biofilms growing without test compounds functioned as controls, while Listerine® (1% v/v) was used for positive controls. Biofilms were tested based on the previous study,[7] with an anaerobic condition. Covers were opened, carefully washed with sterile aquadest twice, followed by washing with 1% glutaraldehyde. Coverslips were coated using carbon tape. After that, coverslips were put in an auto fine coater and analyzed by SEM 6400.[11,12]

Statistical analysis

Based on the results of this study, data were analyzed using (the Statistical Package for the Social Sciences) SPSS statistics for windows, version 16.0 (SPSS Inc., Chicago, USA). Statistical significance of the data was determined using one-way ANOVA, followed by posthoc Bonferroni tests. Differences were considered significant with $P < 0.05$.

RESULTS

Determination of MIC_{50} of C-10 massoia lactone for planktonic microbial growth

The MIC_{50} was determined to analyze the activity of C-10 Massoia lactone against *S. sanguinis, S. mutans, L. acidophilus,* and *A. viscosus.* Listerine® was used as an active positive control that was active compared with DMSO. The results showed that C-10 Massoia lactone inhibited the planktonic growth of *S. sanguinis, S. mutans, L. acidophilus,* and *A. viscosus* at different concentrations of C-10 Massoia lactone [Table 1]. The strongest effect was shown against *S. mutans*. In the results, the effect of C-10 Massoia lactone against *S. sanguinis, S. mutans, L. acidophilus,* and *A. viscosus* was a significant inhibition with $P < 0.05$. Strains were less inhibited compared to the positive control and were significantly inhibited at concentrations of 1% v/v C-10 Massoia lactone for *S. sanguinis, S. mutans, L. acidophilus*, and *A. viscosus*.

Effect of C-10 massoia lactone against mono-species and polymicrobial

The biofilm formation inhibition assay of C-10 Massoia lactone towards biofilm formation was performed at 24 h and 48 h. The effect of C-10 Massoia lactone on biofilm formation of polymicrobial in the intermediate phase was more effective than in the mature phase [Table 1].

The biofilm degradation assay of C-10 Massoia lactone degradation activity towards oral polymicrobial was dose-dependent. The degradation effect increased when the concentration of C10-Massoia lactone increased. In biofilm degradation, C-10 Massoia lactone 1% showed higher activity against polymicrobial biofilms than Listerine® in the intermediate and mature phases [Figure 1].

Scanning electron microscope studies

After treatment with C-10 Massoia lactone, we found that there was clear evidence for the presence of *S. mutans* necrotic cells [Figure 2]. We observed the degradation of *L. acidophilus* EPS [Figure 3], and the degradation of *A. viscosus* EPS [Figure 4]. Our results also showed the capability of C-10 Massoia lactone in degrading EPS of the tested oral polymicrobial [Figure 5].

DISCUSSION

An imbalance in oral polymicrobial can lead to several diseases caused by pathogenic microbes such as *S. sanguinis*, *S. mutans* and *A. viscosus*. Polymicrobial biofilms involve interactions between microbial species.[13] Besides their pathogenetic potential, they can cause dysbiosis and inflammation. We used Listerine® as a positive control treatment showing an $MBIC_{50}$ towards *A. viscosus* at C-10 Massoia lactone 1% v/v both in the intermediate and mature phases [Table 1]. In this study, we tested C-10 Massoia lactone for its effects on mono-microbial and polymicrobial in order to determine its efficacy and identify indications of specificity.

In the previous study, C-10 Massoia lactone was shown to be active as an anti-planktonic and antibiofilm agent against *Staphylococcus aureus*, *Candida albicans*, and *Pseudomonas aeruginosa*.[14] Nanoemulsions with *Massoia aromatica* oil showed stronger antibacterial and antibiofilm activity on *S. aureus* and *P. aeruginosa* as compared to other essential oils.[15]

This study showed that C-10 Massoia lactone is a potential candidate for antibiofilm treatment. The mechanism of this lactone against microbial biofilms is not fully understood. It was suggested it is related with its disruptive function in microbial membranes.[16] C-10 Massoia lactone can penetrate biofilms through the polysaccharide and lipid matrix of a biofilm.[17]

Our results showed that C-10 Massoia lactone inhibited planktonic growth of *S. mutans* more than other

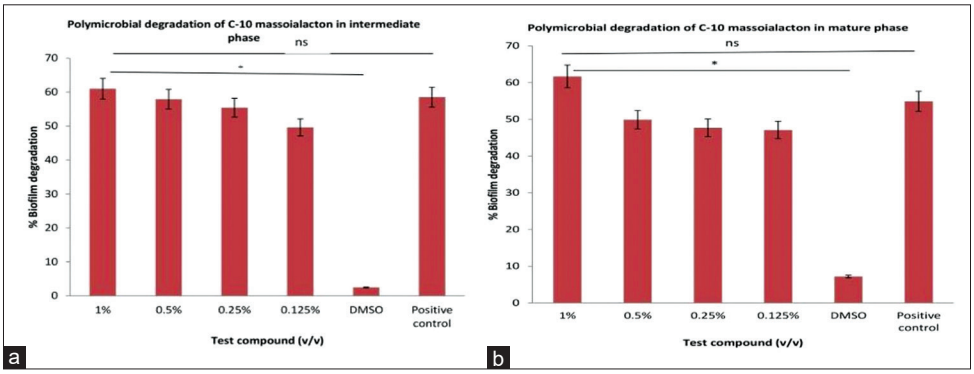

Figure 1: Effect of C-10 Massoia lactone on biofilm degradation of polymicrobial for intermediate phase: (a), mature phase; (b) biofilm, statistical significance of difference was determined by one way ANOVA with *post hoc* Bonferroni. ns: not significant; $*P < 0.05$

Table 1: Minimal inhibitory concentrations$_{50}$, minimum biofilm inhibitory concentration$_{50}$, and minimum value of biofilm eradication concentration$_{50}$ of C-10 massoia lactone

Oral bacterial tested	Planktonic anti-bacterial activity ($PMIC_{50}$) in % v/v*	Anti-biofilm formation activity ($MBIC_{50}$) in %*		Biofilm breakdown activity ($MBEC_{50}$) in % v/v*	
		Intermediate phase	Mature phase	Intermediate phase	Mature phase
Streptococcus sanguinis	1	-	-	0.5	-
Streptococcus mutans	1	-	-	0.25	1
Lactobacillus acidophilus	1	-	-	1	1
Actinomyces viscosus	1	-	-	0.125	0.125

MBIC: Minimum biofilm inhibitory concentration, MBEC: Minimum value of biofilm eradication concentration, PMIC: Planktonic minimum inhibitory concentration

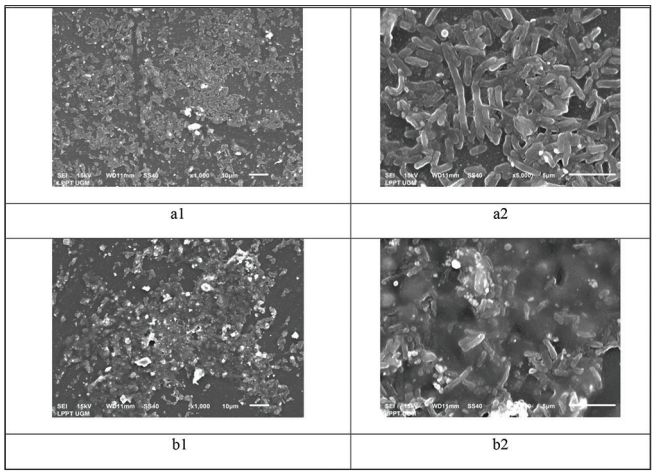

Figure 2: Biofilm eradication activity of C10 Massoia lactone against *S. mutans* ATCC 10566 mature biofilm monitored by scanning electron microscope; (a) cells treated with C10 Massoia lactone at minimum value of biofilm eradication concentration (0.25%); 1: magnification ×1000; 2: magnification ×5000; (b) control (untreated) cells; 1: magnification ×1000; 2: magnification ×5000

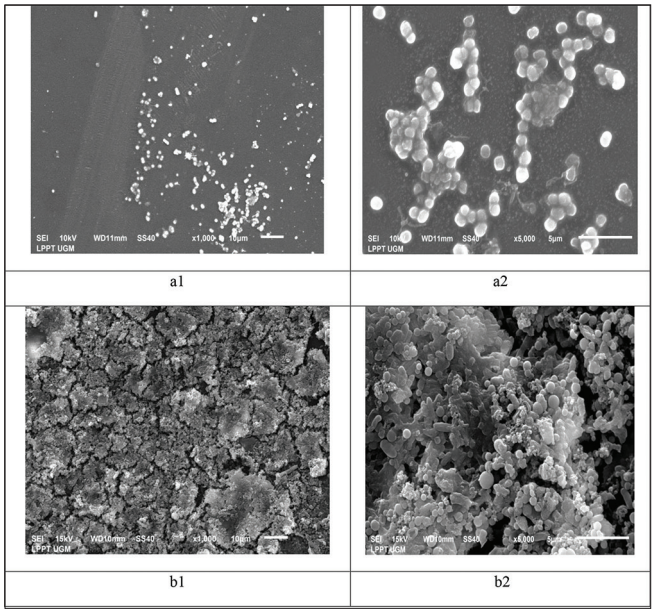

Figure 4: Biofilm eradication activity of C10 massoia lactone against *A. viscosus* ATCC 15987 mature biofilm monitored by scanning electron microscope; (a) cells treated with C10 Massoia lactone at MBEC (1%), 1: magnification ×1000; 2: magnification ×5000; (b) control (untreated) cells; 1: magnification ×1000; 2: magnification ×5000

bacteria [Table 1]. Concerning the effect on mono-species biofilm degradation by C-10 Massoia lactone, we showed that *A. viscosus* was the most affected compared to other bacteria. *A. viscosus* can cause dental caries in the root of teeth.[18,19]

We reported here that C-10 Massoia lactone had a stronger inhibition of polymicrobial biofilms than on mono-species (*S. sanguinis, S. mutans, L. acidophilus,* and

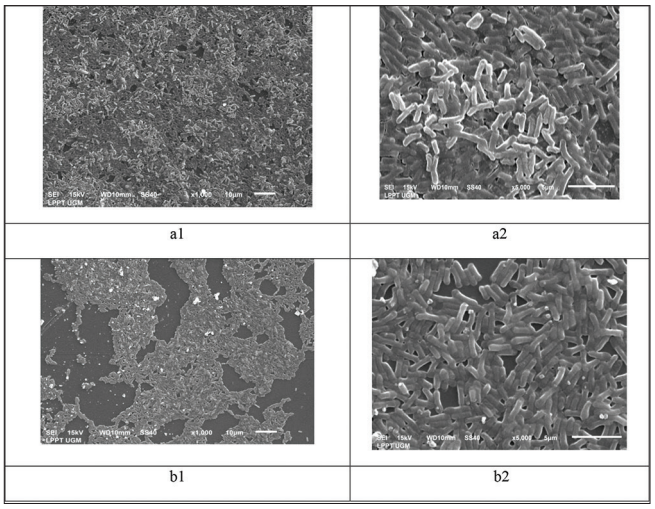

Figure 3: Biofilm eradication activity of C10 massoia lactone against *L.acidophilus* ATCC 4356 mature biofilm monitored by scanning electron microscope; (a) cells treated with C10 Massoia lactone at minimum value of biofilm eradication concentration (1%), 1: magnification ×1000; 2: magnification ×5000; (b) control (untreated) cells; 1: magnification ×1000; 2: magnification ×5000

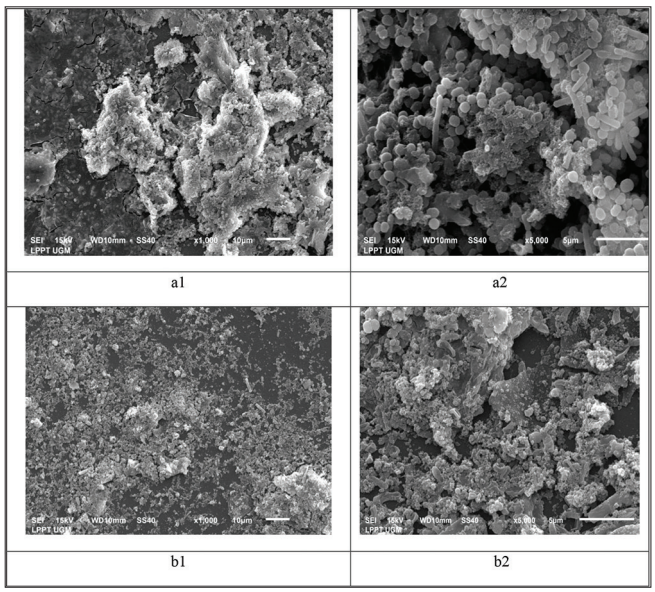

Figure 5: Biofilm eradication activity of C10 Massoia lactone against polymicrobial mature biofilm monitored by scanning electron microscope; (a) cells treated with C10 Massoia lactone at MBEC (1%), 1: magnification ×1000; 2: magnification ×5000; (b) control (untreated) cells; 1: magnification ×1000; 2: magnification ×5000

A. viscosus) biofilms. The SEM results of this study indicated that C-10 Massoia lactone caused leakage of EPS.

We evaluated the antibiofilm potencies of C-10 Massoia lactone at different phases of biofilm formation and degradation. The degradation effect of the test compound was higher than the inhibition effect. $MBIC_{50}$ and Minimum value of biofilm eradication concentration ($MBEC_{50}$) of the test compounds against the tested polymicrobial were much higher in the

mature phase than the intermediate phase of biofilms. This is because the mature biofilms are better protected from the stressful environment than intermediate biofilm.[20]

C-10 Massoia lactone can be developed as a new antibiofilm agent to treat malignant oral biofilm microorganisms. Considering that Massoia lactone is a major constituent of food additives, we expect that further studies will show that moderate concentrations of C-10 Massoia lactone can be a safe drug for treatments against oral biofilms.

CONCLUSIONS

C-10 massoia lactone can degrade polymicrobial biofilms of *S. mutans*, *S. sanguinis*, *L. acidophilus* and *A. viscosus*. This compound can destroy the extracellular polymeric substances of polymicrobial biofilms. Therefore, it can be developed as new antibiofilm candidates against polymicrobial oral biofilms.

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Conflicts of interest

There are no conflicts of interest.

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