INNOVARE JOURNAL OF LIFE SCIENCE

Vol 6, Issue 4, 2018

ISSN - 2321-550X

Research Article

INHIBITION OF *STREPTOCOCCUS MUTANS* ADHERENCE AND BIOFILM FORMATION ACTIVITIES FROM *MELASTOMA MALABATHRICUM* SUBFRACTION

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Received: 13 August 2018, Revised and Accepted: 15 September 2018

ABSTRACT

Objectives: The objectives of the study were to determine antibacterial, anti-adherence, and antibiofilm activities of *Melastoma malabathricum* stem bark acetone extract (MMSBAE) subfraction against *Streptococcus mutans*.

Methods: Fraction 9 (F9) from MMSBAE was subfractionated by thin-layer chromatography (TLC) and analyzed for antibacterial activity against *S. mutans* by TLC-bioautography. Subfraction 12 (SF12) was isolated from F9 followed by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values.

Results: MIC and MBC values were 10 mg/mL and 160 mg/mL, indicating bacteriostatic property of SF12. Time-kill assay analysis confirmed bacteriostatic property of SF12 against *S. mutans*. Crystal violet staining and glass surface assays were used to determine anti-adherence and antibiofilm activities. Concentrations produced 50% reduction in anti-adherence and antibiofilm activities were 40 mg/mL and 20 mg/mL, respectively. Scanning electron microscopy was performed to visualize the effect of SF12 on *S. mutans* biofilm structure. SF12 was found to lyse biofilm formation on treated bacteria indicating powerful anticariogenic potential against *S. mutans*. Analysis by quantitative real-time polymerase chain reaction revealed SF12 at MIC value downregulated biofilm formation genes such as *gbp*A, *brp*A, *gtf*C, and *com*DE.

Conclusion: SF12 showed bacteriostatic activities against *S. mutans* by inhibiting adherence and biofilm activities.

Keywords: *Streptococcus mutans*, Subfraction, *Melastoma malabathricum* stem bark acetone extract, Antibiofilm activity, Anti-adherence activity.

INTRODUCTION

Streptococcus mutans is the main causative agents of dental caries by biofilm formation and increase adherence activities [1]. Dental caries problem arised due to resistance to antibiotic and antiseptic of the causative bacteria. Besides, some mouthwashes used to prevent dental caries contained high concentration of alcohol and can lead to oral cancer. Shekar *et al*. reported that natural products are a good alternative to control oral diseases such as dental caries and periodontal disease [2]. It was suggested that natural products have minor side effects and more importantly able to avoid development of primary or secondary resistance to the drug during therapy.

Melastoma malabathricum (Melastomataceae) or senduduk are used widely in traditional medicine including to relieve toothaches. Stem bark acetone extract of *M. malabathricum* stem bark acetone extract (MMSBAE) was reported previously to possess antibiofilm and anti-adherence activity against *S. mutans* [3]. MMSBAE affects *S. mutans* cell membrane and wall structure which eventually leads to cell death. Fraction 9 (F9) from MMSBAE have been reported to show a large inhibition zone against *S. mutans* by thin-layer chromatography (TLC)-bioautography [4]. Therefore, in this study, a closer look of this subfraction was done, and the mode of action was evaluated especially on the antibiofilm and anti-adherence activities.

METHODS

Preparation of subfraction 12 (SF12)

Preparation of F9 was done following method describe by Hanafiah et al., [4]. F9 was further fractionated to 13 subfractions. Further separation of all the fractions was done by TLC using *n*-hexane:acetone (70:30).

TLC-bioautography

Antibacterial activity against *S. mutans* ATCC 25175 of subfractions from F9 was determined by TLC-bioautography according to Alwash *et al.* [5]. A subfraction designated as SF12 showed clear, unstained spot when 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added. Identification of SF12 bioactive compound was done by gas chromatography-mass spectrometry (GC-MS) using similar protocol explained in Hanafiah *et al*. [3].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

MIC and MBC values were determined according to Alwash *et al*. [5]. A total of 100 µL of *S. mutans* at 10^8 colony-forming unit (CFU)/mL were added to a series of SF12 concentrations (2.5–160 mg/mL) diluted in Mueller-Hinton broth (MHB) to a final volume of 200 µL/well. Dimethyl sulfoxide (DMSO) 10% (v/v) was used as negative control and penicillin at 0.004–0.06 mg/mL was used as positive control. Cultures were incubated at 37°C in anaerobic condition for 24 h. Determination of MIC value was done by observing the lowest concentration which inhibited visible growth of bacteria. Then, it is followed by determination of MBC by subculturing a 5 µL aliquot from MIC value onto nutrient agar and incubated at 37°C for 24 h. MBC was defined as the lowest concentration with no growth on agar plates.

Time-kill assay

The assay was done to determine the bacterial growth according to Raja *et al*. [6]. A total of 200 µL *S. mutans* at ~10^8 CFU/mL were added to 20 mL of MHB in conical flasks containing SF12 at different concentrations (1.25–40 mg/mL). The flasks were incubated at 37°C overnight. A total of 100 µL aliquot were removed from the flasks at several time points

(0, 1, 2, 4, 6, 8, 10, and 24 h) followed by streaking on Tryptic Soy Agar in triplicate for determination of viable bacteria count. Killing curve was plotted as CFU (log_{10} CFU/mL) versus time over 24 h period.

Anti-adherence and antibiofilm formation assays

Biofilm formation and adherence assays were done by following Khan *et al*. [7]. Briefly, 50 µL of SF12 at different concentrations (5–80 mg/mL) was added to *S. mutans* (10^8 CFU/mL) well. Negative control contained *S. mutans* treated with DMSO, while positive control was *S. mutans* treated with penicillin (1 mg/mL). After inoculation, all plates were incubated in an anaerobic incubator at 37°C for 24 h. Biofilm formation was determined by crystal violet assay [3]. For adherence assay, SF12 (5–80 mg/mL) was added to brain heart infusion broth (BHIB) containing *S. mutans* (OD595 ≈ 1) and 0.25% sucrose in a glass tube. All tubes were inclined at 30° and incubated at 37°C for 24 h. Turbidity of cells adhered to the glass slide was determined by spectrophotometer at 600 nm. Tests were performed in triplicate.

Scanning electron microscopy (SEM)

A total of 100 µL of SF12 (10 mg/mL) were added to 5 mL of BHIB containing *S. mutans*, then incubated at 37°C for 24 h. Cultures were plated on BHI agar containing 10% sucrose and incubated for 24 h at 37°C. Negative control of this analysis was non-treated bacteria. Agar was cut into 5 mm × 5 mm and washed with phosphate buffer (pH 7.1) twice. Then, agar was dried and treated with 4% formalin. Dehydration of samples was done by incubating samples in increasing percentage of alcohol (33–99%). Agar was finally coated with gold before observation using SEM.

Real-time polymerase chain reaction (RT-PCR)

Isolation of RNA from *S. mutans* treated with SF12 at MIC value and SF12 non-treated was carried out using the SV total RNA isolation system (Promega, USA), according to the manufacturer's protocol. Following purification, the RNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Complimentary DNA (cDNA) was synthesized using ImProm-II™ reverse transcription system (Promega, USA). Standard reaction mixture with a total volume of 20 mL for RT was prepared to contain 10 mM dNTPs, 25 mM $MgCl_2$, RNAase inhibitor, RT 200 U, oligo-dt 0.5 µg, and 1 mg total RNA from respective samples. First strand complementary DNA (cDNA) was amplified following incubation for 90 min at 42°C and 15 min at 72°°C. The cDNA samples were stored at −20°°C until further use. RT-PCR was done using *SensiFAST™ SYBR* and *Fluorescein kit* (Bioline, USA). Primers *gbp*A, *brp*A, *gtf*C, and *com*DE were used following the suggestion in Hasan *et al*. [8]. PCR condition consists of cDNA denaturation at 95°°C for 2 min, 40-cycles of amplification consisting of 5 s denaturation at 95°°C, annealing, and extension at 60°°C for 15 s. The expression levels of biofilm genes were normalized against *S. mutans* 16S rRNA as a reference gene [8]. The relative quantification model by Livak and Schmittgen was used for determination of mRNA fold change after normalization to internal control [9].

Statistical analysis

The effect of SF12 on biofilms and adherence was analyzed statistically using Students *t*-test. Treatments were considered significantly different if $p<0.05$.

RESULTS

Antibacterial activity determination

F9 was reported by Hanafiah *et al*. as the most active fraction in MMSBAE in inhibiting *S. mutans* [4]. Further fractionation of F9 by HPLC eluted 13 SF with SF12 showed clear inhibition spot against *S. mutans* by TLC-bioautography (Fig. 1). MIC value for SF12 was 10 mg/mL while MBC was 160 mg/mL.

Time-kill assay curve

Fig. 2 showed time-kill assay curve of SF12 against *S. mutans*. From this assay, SF12 displayed bacteriostatic activity due to reduce colony number of <3 log_{10} of CFU from the initial inoculum following incubation for 10 h with SF12 at 10–40 mg/mL concentrations. No growth inhibition was determined at 1.25–5 mg/mL of SF12. Initial exposure to SF12 at 2.5 mg/mL and 5 mg/mL caused no *S. mutans* growth, but re-growth was detected after 4 h of treatment.

Anti-adherence and antibiofilm activities

Fig. 3 showed significant ($p<0.05$) anti-adherence activity of SF12 at all tested concentrations toward *S. mutans* in a dose-dependent manner. Biofilm formation of *S. mutans* reduced significantly in concentration-dependent manner when it treated with SF12 (Fig. 4). Treatment with 80 mg/mL of SF12 inhibited 70% biofilm formation compared to only 20% at MIC value (10 mg/mL). DMSO did not inhibit biofilm formation of *S. mutans,* while penicillin reduced 85% of biofilm formation when compared to non-treated bacteria.

Morphological change of SF12 toward *S. mutans*

Fig. 5a showed SF12 at MIC value disrupted *S. mutans* chain formation causing scattered cells and loss of aggregation. Changes in the structure of treated bacteria were also noted from coccus to elongated and ellipsoid. Treated cells have rough surfaces, and the exopolysaccharide matrix was almost absent suggesting reduction in synthesis peptidoglycan. In comparison, non-treated bacteria surfaces have smoother coccus shaped and aggregated within intact exopolysaccharide matrix (Fig. 5b).

Expression of *gbp*A, *brp*A, *gtf*C, and *com*DE genes

RT-PCR analysis showed SF12 downregulated the expression of all selected biofilm genes: *gbp*A, *brp*A, *gtf*C, and *com*DE (Table 1). The

Fig. 1: Subfraction 12 inhibition zone on thin-layer chromatography-bioautography analysis

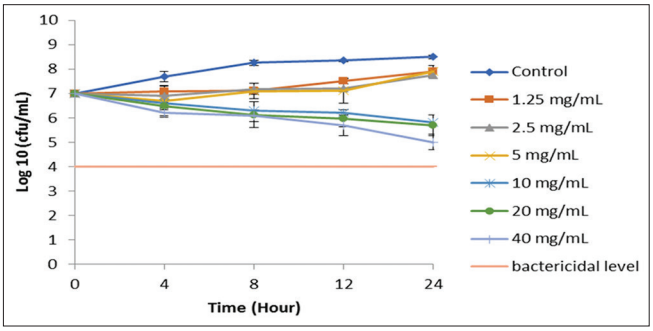

Fig. 2: Time-kill assay curve of subfraction 12 with various concentrations against *Streptococcus mutans*. Non-treated bacteria served as control. Each time point represents the mean of three different experiments performed in triplicate. A number of treated *S. mutans* were significantly lowered ($p<0.05$) when compared to a number of non-treated *S. mutans*

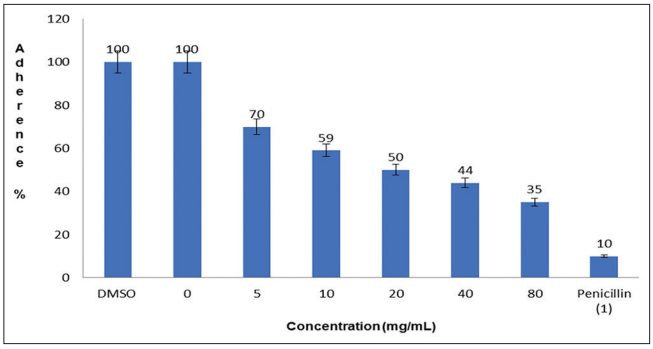

Fig. 3: Adherence inhibition activity of subfraction 12 against *Streptococcus mutans.* All concentrations tested showed significant (p<0.05) decrease in biofilm formation compared to control

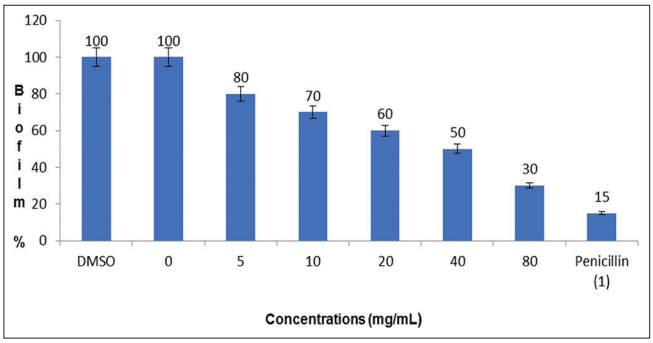

Fig. 4: Biofilm inhibition activity of subfraction 12 against *Streptococcus mutans*. All concentrations tested showed significant (p<0.05) decrease in biofilm formation compared to control

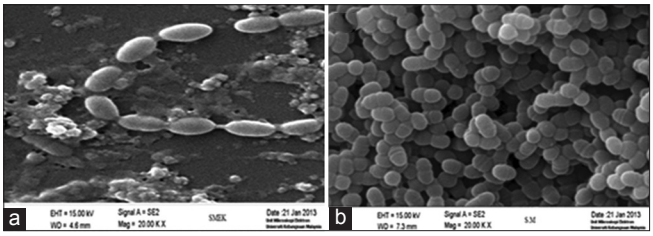

Fig. 5: *Streptococcus mutans* and biofilm structures when treated with subfraction 12 (SF12) at 10 mg/mL (a) and not treated with SF12 (b) image from scanning electron microscopy at 2,00,000 × magnification

Table 1: Relative fold change in gene expression of *S. mutans* biofilm-related genes in cells treated with SF12 (10 mg/mL) normalized against *S. mutans* 16S rRNA as reference gene

Gene	Mean relative gene expression fold change
*gbp*A	0.658±0.11
*brp*A	0.189±0.09
*gtf*C	0.556±0.10
*com*CDE	0.297±0.13

SF12: Subfraction 12, *S. mutans: Streptococcus mutans*

regulation expression level of those genes was important to determine the mechanism of action of SF12 on biofilm formation and quorum sensing (QS) process.

DISCUSSION

According to NCCLS, antibiotics with MBC value which equal to or >4 times MIC value can be considered as bacteriostatic. SF12 showed bacteriostatic property in contrast to the antibacterial activity of crude MMSBAE [3]. According to Pankey and Sabath [10], relying on the determination of MIC and MBC values only to confirm antibacterial activities may not be accurate. Therefore, time-kill assay analysis was done to confirm SF12 bacteriostatic activity of *S. mutans*. According to NCCLS [11], bactericidal activity can be defined as an antibacterial agent which reduced CFU >3 $\log_{10}$ from the initial inoculum. Bacteriostatic activity was defined when the antibacterial agents able to reduce <3 $\log_{10}$ of CFU from the initial inoculum. This observation confirms the bacteriostatic nature of SF12 as determined from MIC and MBC values.

The initial stage of biofilm formation was adherence of bacteria. According to Sandasi *et al*., herbs and plant extracts reduced surface adherence and caused detachment of bacteria followed by antibiofilm activity in *Listeria monocytogenes* [12]. In this study, pre-treatment of cells with SF12 reduced adherence activity, and this was followed by inhibition of biofilm formation in a concentration-dependent manner. However, MMSBAE reduced biofilm formation of *S. mutans* at lower concentration compared to SF12 [3]. For example, MMSBAE reduced 50% biofilm formation at 1.88 mg/mL compare to SF12 which reduced 50% biofilm formation at 40 mg/mL. To further understand this observation, a morphological study of SF12 treated bacteria was performed by SEM. Bacteria was scattered and loss of aggregation due to activity of SF12 on membrane cell. SF12 contained antibacterial properties and disrupted the chain formation in *S. mutans*.

Streptococcal cell wall was made up of four major polymers including peptidoglycan, protein, group and type-specific polysaccharides, and glycerol form of teichoic and lipoteichoic acids [13]. From GC-MS result, SF12 was determined to contain a-amyrin, g-sitosterol, stearic acid, and hexacosanoic acid (raw data not shown). Sitosterol has been proven to possess antimicrobial property [3,14]. Hexacosanoic acid also known as cerotic acid is a long chain saturated fatty acid with not much report in antibacterial activity. However, according to Giacaman *et al.,* stearic acid does not cause a reduction in *S. mutans* colony and insoluble extracellular polysaccharide production [15]. To date, there are no reports on a-amyrin in antibacterial activity. Reduced phytochemical content in SF12 compared to MMSBAE might explain the loss in bactericidal activity but retained bacteriostatic properties. In addition, the anti-adhesiveness property of SF12 shown in this study is the main bacteriostatic strategy against *S. mutans.* This conclusion can be made from scientific evidence that phytochemicals can exert significant multiple anti-streptococcal effects and apart from their bactericidal effect as reviewed in Abachi *et al.* [16]. To further elucidate the molecular mechanism of SF 12 toward *S. mutans*, the effect on expression of biofilm genes was determined by RT-PCR.

RT-PCR analysis revealed that genes involved in biofilm and QS were downregulated when *S. mutans* treated with SF12. The *gbpA* gene was known to be involved in glucose binding protein during the fermentation of sucrose [1]. As for *brp*A, it plays a critical role in biofilm formation and the structural integrity. Depression of these genes will lead to impaired acid tolerance and major structural defects in biofilm formation and integrity, resulting in impaired virulence expressions. Reduction in the expression of these genes supported the SEM observation in SF12 treated cells where exopolysaccharide matrix was found to be reduced.

The *gtf*C gene encodes for glucosyltransferases catalyzes the synthesis of water-soluble and water-insoluble glucan from sucrose [8]. Reduction in the expression of this gene with several other genes will suppress the series of cascades involved in biofilm formation, cell wall integrity, adhesion promotion, and surface biogenesis. The regulatory gene *com*DE is a part of the quorum-sensing cascade of *S. mutans*. Downregulation of this gene attenuated the internal communication system utilized by the bacteria to alter their gene expression at a critical cell density, which may lead to cell death [17].

A lot of resistant bacteria have been reported which lead to an increment of caries incident [18]. As a strategy to overcome this problem,

phytochemicals have been suggested as a new source to interfere with bacterial signaling pathways through QS and perhaps as chelating agents and efflux pump inhibitors [19]. There are lots of recent studies reported medicinal plant extract to possess anti-QS, anti-adherence, antibiofilm, antiproliferative, and antibacteria properties against oral bacteria [20-23]. SF12 has been shown to display the characteristics in anti-adherence and antibiofilm activities which may contribute to the use of *M. malabathricum* in controlling dental caries.

CONCLUSION

SF12 showed bacteriostatic activities against *S. mutans* by inhibiting biofilm formation and adherence activities in dose-dependent manner ($p<0.05$). Downregulation of biofilm producing genes such as *gbp*A, *brp*A, *gtf*C, and *com*DE contributed to the reduction in QS, adherence activities which leads to an inhibition in biofilm formation and disruption in the structural integrity.

ACKNOWLEDGMENTS

This work was supported by research grants from Universiti Sains Islam Malaysia (PPP-FPG-10516-00), Universiti Kebangsaan Malaysia (BKBP-K006401) and Ministry of Higher Education Malaysia (FRGS-FPG-51717-50).

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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