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Article

Anti-Bacterial Activities of *Melastoma Malabathricum* Stem Bark Fractions against *Streptococcus Mutans*

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***Abstract*— The objective of this study is to isolate and identify the bioactive compounds that possess antibacterial activities from *Melastoma malabathricum* stem bark acetone extract (MMSBAE) against *Streptococcus mutans*. *M. malabathricum* is widely used in the Southeast Asia to treat many ailments. A total of 12 fractions was purified by vacuum liquid chromatography (VLC) and further analysed by TLC-bioautography to determine antibacterial activities. TLC-bioautography showed that fraction 9 possesses antibacterial activities against *S. mutans*. Identification of fraction 9 had been done by GCMS and revealed 21 compounds. Some of the compound were important as agent pharmaceutical such as α-amyrin, β-sitosterol, hexadecenoic acid, stearic acid and hexacosanoic acid. Crystal violet and glass surface assay were used to determine anti-biofilm and anti-adherence activity, respectively. The concentrations of fraction 9 that produce 50% reduction in anti-biofilm and anti-adherence activities were 5 mg/mL and 2.50 mg/mL, respectively. Scanning electron microscopy (SEM) was performed to visualize the effect of the fraction 9 on biofilm structure of *S. mutans*. SEM analysis showed lysed biofilm were found on treated cells. These results indicated that this fraction possesses a powerful anti-cariogenic potential against *S. mutans*.**

Keywords*— *Melastoma malabathricum; Streptococcus mutans.

I. INTRODUCTION

Across Southeast Asia, *Melastoma malabathricum* is a well-known medicinal plant used by different communities. In general, various parts of this plant (e.g. leaves, roots, and barks) are used in Malay, Indonesian, and Indian medicine to treat diarrhea, dysentery, leucorrhoea, haemorrhoids, wounds, confinement infections, toothache, stomach ache, flatulence, sore legs, and thrush. The antimicrobial activities of *M. malabathricum* against pathogenic bacteria have been previously documented [1]. However, the studies on the anti-biofilm of anti-adherence activities of *M. malabathricum* are limited, though such studies will provide adequate information on the capability of an extract to control microbial manifestations associated with living or abiotic surface.

Streptococcus mutans is the main cariogenic bacteria in humans. These bacteria are moderately resistant to antibiotics. This drawback justifies for further research and development of natural antibacterial agents that are safe for the host and specific for oral pathogens [2]. Many members of the genus *Streptococcus* that cause infections in humans use quorum-sensing systems to regulate several physiological properties, including incorporating foreign DNA, tolerating acids, forming biofilms, and becoming virulent [3], [4]. *S. mutans* is a bacterium that has evolved to depend on a biofilm lifestyle for survival and persistence in its natural ecosystem. The bacteria are assembled as communities attached to dental surfaces and forms matrix-embedded biofilms. Adherence is the initial step in the formation of biofilm communities [5]. Previous research has indicated dental plaque is an example of a biofilm that has a primary role in pathogenesis of disease [4]. As a primary bacterial agent of dental caries, the mechanisms by which *S. mutans* adheres to tooth surfaces are important targets for anti-cariogenic intervention.

Most antibiotics that are currently used against oral bacteria such as *S. mutans* have side effects such as vomiting, diarrhea and tooth staining [6], [7]. Bacteria are resistant to most antibiotics that are used to treat oral infections such as penicillin, cephalosporin, erythromycin, tetracycline and metronidazole [8]. Antibacterial agents used in the prevention and treatment of oral diseases such as

cetylpyridinium chloride, chlorhexidine, amine fluorides and product containing them are reported to exhibit toxicity, cause staining of teeth and have been linked to oral cancer [9].

The objective of this study is to isolate and identify the bioactive compounds that possess antibacterial activities from *M. malabathricum* stem bark acetone extract (MMSBAE) against *S. mutans*. At the end of the study, we want to indicate whether the compound in *M. malabathricum* possesses a powerful anti-cariogenic potential, especially to treat oral diseases such as caries.

II. Materials and Method

A. Plant Collection and Extraction

M. malabathricum stem bark were collected in Pedas, Negeri Sembilan. The plants were dried in 40˚ C, then ground to form powder and stored in a closed container.

B. Preparation of Extract and Isolation Active Compound

800g of stem bark were extracted with 1L acetone. The mixture was vigorously shaken for 2 hours on an orbital shaker (LM-530RD, Taiwan) and the extract was filtered using Whatman No. 1 filter paper. The collected solvent filtrate was evaporated using a rotary evaporator (Laborota 4000, Germany).

C. Isolation of Active Compound

MMSBAE (1.1g) was fractionated by vacuum liquid chromatography (VLC) on silica gel 60 (230–400 mesh) using *n*-hexane and acetone solvents with ratios *n*-hexane: acetone of 10:0, 9:1, 8:2, 7:3, 6:4 and 5:5. Bacterial activity was determined by thin layer chromatography bioautography. The method of TLC-bioautography was followed by references [10]. Component of fractions was separated by TLC solvent system with *n*-hexane: acetone ratio of 70:30. TLC plate was dipped in a suspension of *S. mutans* in Brain Heart Infusion (BHI) broth and incubated at 37°C under anaerobic condition for 4 hours. The TLC plate was then sprayed with MTT and incubated for 8 hours to produce inhibition zone.

D. Gas Chromatography Mass Spectrometry (GC -MS) Analysis.

A bioactive component of fraction 9 was determined by gas chromatography mass spectrometry (GC-MS) (Shimadzu, model QP5050A) equipped with a HP5 (50m) capillary column. Helium (1.0ml/min) was used as the carrier gas. The temperature was programmed from 60°C to 280°C at the rate of 60°C for 2 minutes and held at 280°C for 12 minutes. Then, the injector was maintained at 245°C and the electron impact ion source was maintained at 295°C. Electron impact spectra were recorded at 70eV. These compounds were identified by comparing the GC retention indices with the mass spectra provided by National Institute Standard and Technology (NIST) database.

E. Determination of Anti-Bacterial Activity

1) Bacterial Strain: Bacterial strain used in this study was bought from ATCC (*S. mutans* ATCC25175), which was grown in Brain Heart Infusion (BHI) Broth (Oxoid, England) at 37ºC. Glycerol stock of the bacteria was kept at -80 ºC.

2) Minimal Inhibitory Concentration (MIC): MIC of fraction 9 against *S. mutans* was determined using a broth microdilution method with modification [11]. The inoculums were prepared in BHI, and the density was adjusted to 0.5 McFarland standard (10^8 CFU ml^{-1}). Fraction 9 was diluted to concentrations ranged from 1 to 10 mg/mL in a series of two-fold dilutions and added to the inoculums BHI. Micro-titre plates were incubated at 37°C in anaerobic condition, and the MIC was recorded after 24 hours. The MIC is the lowest concentration of the compound at which the microorganism tested does not demonstrate visible growth.

3) Anti-Biofilm Formation Assay: Anti-biofilm assay was performed using the method from references [12] with modification. Biofilm assay was carried out using sterile polystyrene 96-well (flat-bottom) cell. The wells were coated with saliva preparations stated by [13]. Overnight culture of *S. mutans* was transferred to preheated BHI broth and grown at 37°C under anaerobic condition to the mid-exponential phase (OD_{600}=1). The cultures were then diluted 1: 100 in preheated BHI media. Two hundred micro litres of the cell suspensions were inoculated into the wells with different concentrations of the fraction 9 (1 to 10 mg/mL). Penicillin was used as the positive control, medium without the fraction as the non-treated control, medium with bacteria inoculums were used as growth control. DMSO had been used as negative control because it is diluent of MMSBAE. After inoculation, all plates were incubated at 37°C for 24 hours anaerobically. The culture medium was then decanted, and the plates were gently washed twice with 200µl with sterile distilled water to remove planktonic and loosely bound cells. The adherent bacteria were stained with 50 µL of 0.1% crystal violet for 15 minutes. After rinsing twice with 200 µL of water, the bound dye was extracted from the stained cells using 200 µl of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 600 nm in a microplate reader (Model 680, Bio-Rad).

4) Anti-Adherence Assay: Adherence assay was done using methods from [12] with modification. BHI broth containing 0.25% (w/v) sucrose fraction 9 (the concentrations ranged from 1 to 10 mg/ mL) was inoculated with *S. mutans*. The mixture was incubated anaerobically at 37°C for 24 hours. The tubes were then gently rotated, and the adhered cells were removed by addition of sodium hydroxide 0.5 mol^{-l}, washed, suspended in saline and estimated by spectrophotometric at 600 nm. Determination was performed in triplicates using cell grown in BHI medium without treatment as growth control.

$$\text{Anti adherence activity\%} = \frac{\text{O.D of adhered cells}}{\text{O.D of total cells}} \text{x100\%} \quad (1)$$

F. Scanning Electron Microscopy (SEM)

Biofilms of *S. mutans* were formed on glass slide (1×1cm) for 24 h. These biofilms were then treated with fraction 9 (5 mg/mL) and incubated further for 24 h. These slides were rinsed with PBS, air dried and treated with 4% glutaraldehyde (Sigma, USA). The treated slides were kept

in dark for 2 h and were successively dehydrated using an increasing proportion of alcohol (33%-99.9%). The samples were air dried, coated with platinum vapor and observed under a scanning laser electron microscope (Jeol 6360A LV, Japan). Biofilms without compound treatment were served as controls.

G. Statistical Analysis

The effect of fraction 9 on biofilms and adherence were analyzed statistically by the Students t-test and treatments were considered significantly different if $P \leq 0.05$.

III. RESULT AND DISCUSSIONS

A. Isolation and Identification of Active Compounds

Twelve fractions were successfully separated by VLC according to different ratios of the solvent system and resulted in different polarities. Solvents system, number of components and R_f of components for each fraction were shown in Table 1. Fractionation had been successfully separated by hexane and acetone. Then, all the fractionation had been further separated by using thin layer chromatography (TLC). The separation had been successfully achieved by using a ratio of 70% n-hexane and 30% of acetone. TLC-bioautography analysis had been done to determine antibacterial activities of the fractions. In TLC-bioautography analysis, the dehydrogenase of living microorganisms converts MTT into intensely coloured formazan. As a result, cream-white spots appeared against a purple background on the TLC plate surface, pointing the presence of antibacterial agents [10].

The TLC-bioautography depicted in Figure 1 showed that fraction 9 was active against *S. mutans*. Two components in fraction 9 showed a large inhibition zones on TLC-bioautography analysis. In this context, fraction 9 was used for further determination of anti-biofilm and anti-adherence activity of *S. mutans*, and to identify the structure of the active compounds via GCMS analysis.

TABLE I
SOLVENT SYSTEM, NUMBER OF COMPONENTS AND R_f VALUES IN EACH OF FRACTIONS SEPARATED BY VLC. THE COMPONENTS WERE SEPARATED BY RATIO 70:30 *n*-HEXANE: ACETONE

Fraction	**Solvent System (*n*-Hexane %: Acetone %)**	**Number of Components**	**R_f Values**
1	100:0	0	0
2	95:5	0	0
3	90:0	3	0.62,0.81, 0.89
4	85:15	7	0.75,0.67,0.59 0.45,0.43,0.37, 0.32
5	80:0	7	0.75,0.67,0.59 0.40,0.37,0.32, 0.29
6	75:25	5	0.41,0.37,0.32, 0.29, 0.14
7	70:30	2	0.35,0.54
8	65:35	2	0.35,0.54
9	60:40	2	0.86, 0.35
10	55:45	3	0.86, 0.48, 0.35
11	50:40	2	0.48,0.35
12	0:100	2	0.48, 0.35

Fig.1 Inhibition zone of fraction 9 on TLC-bioautography analysis

GCMS analysis of compounds available in fraction 9 is presented in Figure 2. GCMS is a combination of two different analytical techniques which are gas chromatography and mass spectrometry. Gas chromatography was used to separate compound in fraction 9. Mass spectrometry acts as a detector that fragments a compound by ionization. The fragments were sorted by mass to form a fragmentation pattern. The pattern was used to identify a characteristic of the compound in fraction 9. Twenty-one compounds were identified from the fraction 9 and 5 compounds have been reported to have antimicrobial properties including α-amyrin, β-sitosterol, hexadecenoic acid, stearic acid and hexacosanoic acid (Table 2). The antimicrobial activities of those compounds against pathogenic bacteria have been demonstrated. α- Amyrin had been reported to possess anti-inflammatory and high anti-oxidant activities [1]. Whereas β-sitosterol have been found to inhibit *S. mutans* adherence [14]. A study in [15] has reported that the fatty acid such as hexanoic, stearic and hexacosanoic acid inhibit growth of oral bacteria such as *S. mutans* and *Streptococcus gordonii*. A study in [16] found that the fatty acid from plant extract possess antibacterial activity against bacteria pathogen.

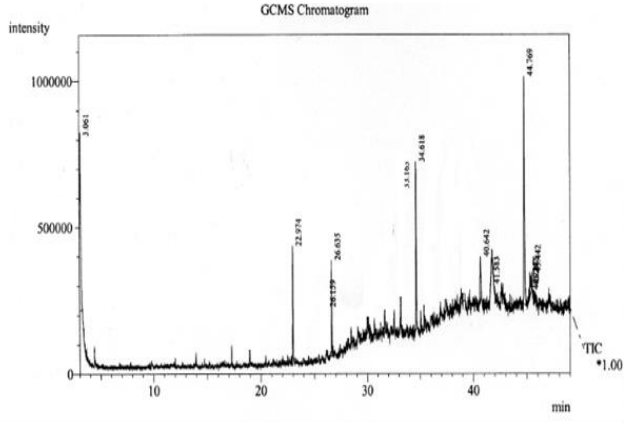


Fig. 2 GC–MS chromatogram of fraction 9 with percentage abundance (parenthesis).

TABLE III
GCMS ANALYSIS OF PHYTOCHEMICALS COMPOUNDS IDENTIFIED FROM FRACTION 9

No	Compound	Molecular Formula	Molecular weight
1	α-Amyrin	$C_{30}H_{50}O$	426.73
2	β-sitosterol	$C_{29}H_{50}O$	414.72
3	Hexadecenoic acid	$C_{16}H_{32}O$	256.43
4	Stearic acid	$C_{18}H_{36}O_2$	284.48
5	Hexacosanoic acid	$C_{36}H_{52}O_2$	396.69

B. Anti-Bacterial, Anti-Biofilm and Anti-Adherence Activities

The MIC of fraction 9 against *S. mutans* was 5mg/mL. The effect of the compound on biofilm produced by *S. mutans* is demonstrated in Figure 3. The concentration that reduced 50% of biofilm formation in *S. mutans* was 5 mg/mL. The inhibitory effect was also found to be in a dose-dependent manner. Negative control (DMSO) did not inhibit biofilm structure and penicillin that serve as a positive control, reduced 15% biofilm at 1 mg/mL. DMSO had been used as negative control because fraction 9 was diluted with DMSO in this test. Although penicillin inhibited biofilm structure better than the fraction 9, but penicillin had been reported resistant to oral bacteria including *S. mitis, S. sangius* and *Peptostreptococcus sp* [8]. Fraction 9 exerted a significant effect ($P < 0.05$) on the inhibition of biofilm by *Streptococcus mutans* at all concentrations.

The effect on adherence of *S. mutans* by different concentrations of fraction 9 is given in Figure 4. The reduction in adherence was found to be in a dose-dependent manner. The concentration that inhibited 50% adherence was 2.50 mg/mL. Significant ($P < 0.05$) reduction in adherence activity by *S. mutans* was observed in all the tested concentration. No reduction in the adherence was found in cell treated with DMSO and untreated cells. Whereas penicillin reduced adherence activity of *S. mutans* to 10% at 1mg/mL.

Inhibition of biofilm formation occurred due to the presence of a glycocalyx (extra polymeric substances) surrounds of biofilm, degradation by antimicrobial agent and efflux pumps that expel drugs from the cells. The negative charge on extra polymeric substances restricted the penetration of molecules by charge attraction, thereby imparting resistance to biofilm formation [17]. Nutrient and oxygen supply also have been reported as factor to inhibit biofilm formation [18]. Anti-biofilm activities of medicinal plants against *S. mutans* have been demonstrated. For example, compounds from cranberry fruit have been reported to reduce biofilm formation produced by *S. mutans* [19] and a novel compound from *Trachyspermum ammi* seeds was also reported as an agent for anti-biofilm against *S. mutans* [12].

The adherence activity of the cells is the initial stage in biofilm formation. The activity requires substances such as nutrients, organic and inorganic molecules. The substances are important for the growth of cells and cell adhesion. Pre-treatment of the cells with plant extracts produces an unfavourable film that promotes detachment, thereby reducing a surface adhesion [20]. Many medicinal plants extracts have been reported to inhibit cell attachment. A study in [21] demonstrated that *Piper betle* and *Psidium guajava* extract have anti-adherence activity of *S. mitis, S. sanguinis* and *Actinomyces sp*. A report in [22] confirmed that high molecular coffee fractions can inhibit the adherence activity of *S. mutans*.

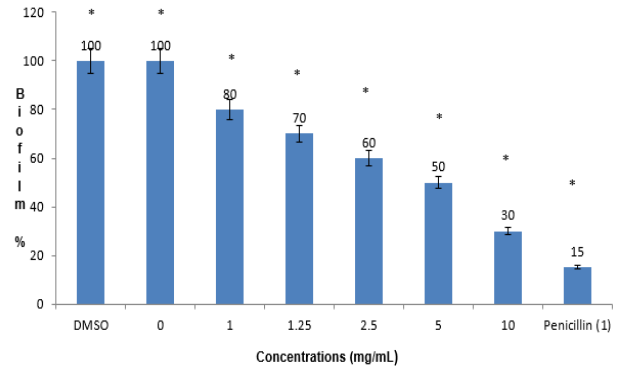


Fig 3. Biofilm inhibition activity of fraction 9 against *S. mutans*. All concentration tested showed significant ($P<0.05$) decrease in biofilm formation compared to control.

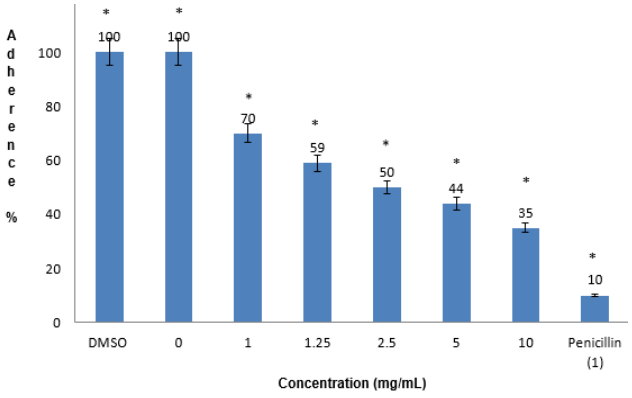
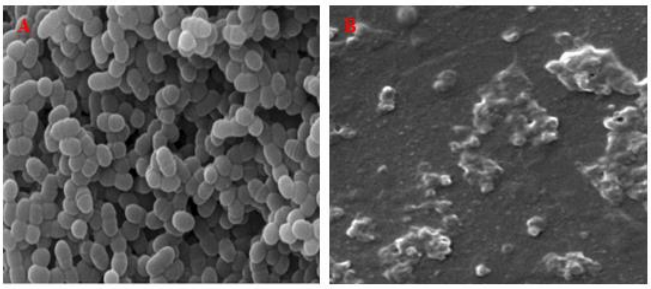


Fig. 4 Adherence inhibition activity of fraction 9 against *S. mutans*. All concentration tested showed significant ($P<0.05$) decrease in adherence activity compared to control.

C. Scanning Electron Microscopy Examination

The biofilm structure in treated and untreated cell by scanning electron microscopy was shown in Figure 5. The cell was observed after 24 hours when fraction 9 was exposed to the cells. Morphology of untreated cells biofilms were normal in round coccus-shaped chain and smooth while entangled in a thick biofilm mass, clumping and aggregate (Figure 5a). The morphology in treated cells was ruptured biofilm, damaged cell walls and demonstrates rough cell features (Figure 5b). The action of natural anti-caries compounds could occur either by inhibition of the bacterial adhesion without killing the bacteria [23] or by destroying the integrity of the cell wall [24]. Biofilm exposed to fraction 9 showed sprinkled and complete loss of aggregates. For this reason, we believe that fraction 9 inhibited the construction of biofilm produced by *S. mutans*.



(a) (b)

Fig. 5 SEM images with 133200x magnification of *Streptococcus mutans* biofilm. (a) Biofilm structure in absence of the fraction. (b) Biofilm structure in presence of the fraction 9.

IV. Conclusions

In summary, a few bioactive compounds such as α-amyrin, β-sitosterol, hexadecenoic acid, stearic acid and hexacosanoic acid have been identified from fraction 9. The bioactive compounds were significantly reduced biofilm formation as well as adherence activity. SEM result shows the morphological cell which has been treated was ruptured biofilm, damaged cell walls and demonstrates rough cell features. These results indicated that this compound possesses a powerful anti-cariogenic potential.

Acknowledgment

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