ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Melastoma malabathricum stem bark acetone extract as an anti-bacterial agent against *Streptococcus mutans*

Rohazila Mohamad Hanafiah[1], Siti Aisyah Abd Ghafar*[1], Nur Syahirah Salehuddin[1], Wan Syaidatul Aqma[2], Nazlina Ibrahim[2]

[1]Department of Basic Science and Oral Biology, Faculty of Dentistry, Universiti Sains Islam Malaysia, 55100 Pandan Indah, Selangor, Malaysia
[2]Department of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Article History:

Received on: 13 Jul 2020
Revised on: 13 Aug 2020
Accepted on: 19 Aug 2020

Keywords:

Anti bacterial agent,
Melastoma malabathricum,
next gene sequencing,
Streptococcus mutans

ABSTRACT

Melastoma malabathricum, also known as 'senduduk' in Malaysia, has been used as traditional medicine for diseases such as toothache, dysentery, haemorrhoids and stomachache. Therefore, the objective of this study is to investigate the biological activity of *Melastoma malabathricum* stem bark extracts (MMSBE) towards *Streptococcus mutans*. This investigation involved a few methods, which at first is the determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). Next is by analyzing the time-kill curve, anti-biofilm activity, scanning electron and transmission electron microscopic analyses. Later, next-generation sequencing (NGS) was done to determine differential regulation genes of treated and non-treated *S. mutans*. Lastly, confirmation of differential regulation genes was done by RT-PCR analysis. As for the results, *M. malabathricum* stem bark acetone extract (MMSBAE) showed the greatest inhibition concentrations towards *S. mutans*, followed by *M. malabathricum* methanol extract (MMSBME). Values of MIC and MBC (MMSBAE) were 1.25 mg/mL and 5 mg/mL. Meanwhile, MIC and MBC values of MMSBME were 5 mg/mL and 40 mg/mL. MMSBAE was chosen to further analyze its anti-bacterial activity against *S. mutans*. Time kill curve analysis found that MMSBAE possessed bacteriostatic properties against *S. mutans*. Besides, SEM and TEM analyses revealed that there were some changes to *S. mutans* cell morphology after treated with MMSBAE while Next gene sequencing analysis revealed significant ($p<0.05$) gene expression with multiple targets by MMSBAE, which caused inhibition of *S. mutans* biofilm formation activity.

*Corresponding Author

Name: Siti Aisyah Abd Ghafar
Phone: +60342891157
Email: aisyahghafar@usim.edu.my

ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v11i4.3801

Production and Hosted by
IJRPS | www.ijrps.com

INTRODUCTION

Streptococcus mutans (*S. mutans*) usually found in the oral cavity, and one of the significant contributors to dental caries (tooth decay). *S. mutans* will cause a breakdown of sugar for energy then produces acidic environment, which will demineralize superficial tooth structure. The conversion disintegrates the tooth coating which then dissolves the calcium molecule thus, creating a hole (Javed *et al.*, 2012).

Although fluoride has been used to prevent dental caries, a recent study showed *S. mutans* were resistance towards fluoride (Liao *et al.*, 2017). Besides that, fluoride was found toxic to human buccal cells if used at higher concentration (Lee *et al.*, 2011). Hence, it is crucial to find alternative medicines such as plant extracts to reduce biofilm formation by *S. mutans*.

Melastoma malabathricum locally known as 'senduduk' is an uncultivated plant mainly found throughout Southeast Asian countries, including Malaysia (Joffrey *et al.*, 2012). *M. malabathricum* root aqueous extract was used to relieve tooth pain and previous study were reported that leaves extract exhibited anti-inflammatory, anti-ulcerogenic and hypotensive effects (Susanti *et al.*, 2007; Zakaria *et al.*, 2006). Other than that, *M. malabathricum* extract possessed anti-bacterial properties against gram-negative bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Vibrio cholerae* (Maji *et al.*, 2010). Several bioactive compounds have been isolated from *M. malabathricum* such as α-amyrin, quercetin, tannins, rutin, terpenes, ethyl ester, γ-sitosterol and fatty acids which possessed anti-bacterial properties (Susanti *et al.*, 2007; Hanafiah *et al.*, 2015). Therefore, the objective of this study is to investigate the biological activity of MMBSAE against *S. mutans* by using anti-bacterial assay and next-generation sequencing (NGS) analysis. To the best of our knowledge, this study represents the most extensive NGS study to date profiling novel *S. mutans* response to MMSBAE.

MATERIALS AND METHODS

Plant material

The stem bark of *M. malabathricum* was collected from an uncultivated area of Bangi, Selangor, Malaysia. The voucher specimen number [Rohazila 2014-1 (UKMB)] was deposited in the herbarium at Universiti Kebangsaan Malaysia for future reference.

Preparation of *M. malabathricum* stem bark extracts and bacterial strain

The stem bark of *M. malabathricum* was dried at room temperature and pounded to a fine powder (Model C14, KesmacSdn. Bhd., Malaysia) then it was stored in black containers. Serial extraction had been done to Stem barks (800 g) with 1 L of *n*-hexane, dichloromethane (DCM), acetone and methanol (System, Shah Alam, Malaysia) (Shai *et al.*, 2008). After that, the extracts were filtered by using Whatman No. 1 filter paper and evaporated it by using a rotary evaporator (Laborota 4000, Germany). Each extract was diluted in 10% DMSO (Systerm, Shah Alam, Malaysia). *S. mutans* strain (ATCC 25175) was used in this study. *S. mutans* were grown on the brain–heart infusion agar (BHIA) and brain–heart infusion broth (BHIB) (Oxoid Ltd., Basingstoke, UK).

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

Minimum inhibitory concentration (MIC) was performed using two-fold serial dilution method in 96 well plates. A hundred microliters of MMSBAE and MMSBME (40.00 to 0.04 mg/mL) were diluted in MHB and added into 96 well plates. *S. mutans* (100 μL) at 10^8 CFU/mL was added into the well to final volume 200 μL/well. Positive and negative control were 10% of DMSO (v/v) and 1 mg/mL of NaF (Sigma-Aldrich, Missouri, USA), respectively. MIC and MBC values were determined by MTT assay after incubation at 37°C under the anaerobic condition for 24 h.

Time-kill analysis of S. mutans to MMSBAE

To determine MMSBAE treatment time, *S. mutans* time-kill curves exposed with MMSBAE were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 1999). *S. mutans* was diluted until reach an optical density at 600 nm (OD600). Then, the cultures (100 μL) were added to the 96-well plate. MMSBAE was added to *S. mutans* (ATCC 25175) to obtain final concentrations from 1.25 mg/mL to 40 mg/mL. Non-treated *S. mutans* was used as a negative control, and broth only was used as inoculum and sterility control. After incubation, the cultures were monitored by spectrophotometrically at OD600 at 4-24 hours time intervals. After that, 50 mL of bacteria were collected every 4 hours and cultured onto BHIA and incubated 24 hours at 37°C. The sample collections had been done until 24 hours.

Biofilm formation assay

Biofilm formation assay was determined by quantitative assay with a 96-well plate, whereby biofilm was stained with crystal violet (Sigma-Aldrich, Missouri, USA). An aliquot of 1 mL *S. mutans* was transferred to 10 mL BHIB and incubated at 37°C until reach mid-exponential phase (OD_{595}=1). Then, the culture was diluted 1:100 in BHIB. A total of 150 μL of bacteria were added to the 96-well plate. After that, MMSBAE (50 μL) at different concentrations from 0.24 to 30 mg/mL was added into the same well. Positive and negative control of this assay were NaF (1 mg/mL) and 10% DMSO. Then, the plate was incubated at 37°C for 24 hours. Biofilm was determined by measuring the absorbance of the samples at 595 nm using a microplate reader (Model 680, Bio-Rad, California, USA).

Scanning electron microscopic (SEM)

S. mutans cultures were grown on BHIA with 10% sucrose. Then, treated with 1×MIC MMSBAE (1.25 mg/mL) and incubated for 4 and 8 hours at 37°C. After that, the agar was cut into 5mm × 5mm and washed two times with PBS (Sigma-Aldrich, Missouri, USA). Next, treated with 4% formaldehyde (Sigma-Aldrich, Missouri, USA) and dehydrated with alcohol and water ascendingly (33-99%). The agar was coated with gold and observation was done via scanning electron microscopic (SEM, Jeol JSM-6700F, Tokyo Japan). Non-treated *S. mutans* were acting as a negative control.

Transmission electron microscopic (TEM)

S. mutans treated with 1×MIC MMSBAE (1.25 mg/mL) were grown on BHIB with 10% sucrose at 37°C for 4 and 8 hours. Then, 1 mL of *S. mutans* were centrifuged at 13000 rpm for 30 minutes using microcentrifuge (Eppendorf, 5415R, Hamburg, Germany) to form a pellet. The pellet was incubated with formaldehyde (4%) for 24 hours and stabilized with osmium tetroxide (Sigma-Aldrich, Missouri, USA). After that, dehydration was done with alcohol and water ascendingly (33-99%). The sample was cut with ultra-microtome and *S. mutans* morphology was observed via transmission electron microscopic (TEM, Jeol, JEM-2100, Tokyo, Japan). Non-treated *S. mutans* were acting as a negative control.

RNA isolation

RNAs isolation was carried out using the SV Total RNA Isolation System, according to the manufacturer's protocol (Promega, Wisconsin, USA). RNA isolation had been done on *S. mutans* treated with MMSBAE (MIC) and non-treated *S. mutans*. Following purification, the RNA concentration had been determined by using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A260/A280 and A260/A230 ratios had been used to confirm RNA isolation were free from contaminants such as proteins, phenol and salts.

Next-generation sequencing (NGS)

Transcription analysis of gene expression was performed in triplicate by Malaysian Genomic Resource Centre Berhad (Lembah Pantai, Malaysia). Sequencing was done towards *S. mutans* RNA treated with MMSBAE. Non-treated bacteria act as a control to compare with treated bacteria. RNAs integrity was measured using Agilent Bioanalyzer RNA Nano Chip (Agilent, California, USA). High-quality RNA was then proceeded with cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen, USA). Produced cDNAs were reduced to small fragments using Covaris S220 (Covaris Inc, Massachusetts, USA). Fragments were connected to Illumina Tru seq adapter and amplified using PCR Tru seq RNA sample preparation kit (Illumina, California USA). The PCR products were sequenced using Illumina Hiseq 2000 (Illumina, California USA). The sequenced data was matched with *S. mutans* NN2025 (AP010655.1), then compared and analyzed using bioinformatics, namely de novo sequencing.

Real-Time PCR

Real-time PCR was done by using *SensiFAST^{TM} SYBR* and *Fluorescein kit* (Bioline, London, UK). Primers used in this analysis are shown in Table 1. Real-time PCR consists of cDNA denaturation at 95 °C for 2 min. Then the amplification step consists of a denaturation step at 95 °C with annealing and extension steps at 60 °C for 15 seconds. Normalization of gene expression had been done by using *S. mutans* 16S rRNA as a reference gene. The relative quantification model by (Livak and Schmittgen, 2001) was used for determination of mRNA fold change after normalization to internal control. Tests were done in triplicate.

RESULTS AND DISCUSSION

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

Four solvents were chosen based on their polarity level. N-hexane and DCM were used to extract non-polar compounds; meanwhile, acetone was used to extract semi-polar compounds and methanol was used to extract polar compounds. Polar solvents have been used to isolate bio active compounds that exhibited anti-bacterial and antiseptic properties such as phenol group (Joffrey *et al.*, 2012).

In this study, the MIC value of MMSBAE against *S. mutans* was 1.25 mg/mL, meanwhile MBC value of MMSBAE was 5 mg/mL. Values MIC and MBC of MMSBME were 5 mg/mL and 40 mg/mL, respectively. MIC and MBC values of NaF against *S. mutans* were 1 mg/mL and 2 mg/mL, respectively. Based on the results, the MBC value of MMSBAE (5 mg/mL) was not more than four times of MIC value (1.25 mg/mL).

Bactericidal activity had been defined if the value of MBC was not more than four times the value of MIC (CLSI, 1999) and this was presumed to tell that MMSBAE is bactericidal agent against *S. mutans*. Therefore, MMSBAE was chosen to further analyze their biological activity against *S. mutans* growth in terms of phenotypic and molecular studies.

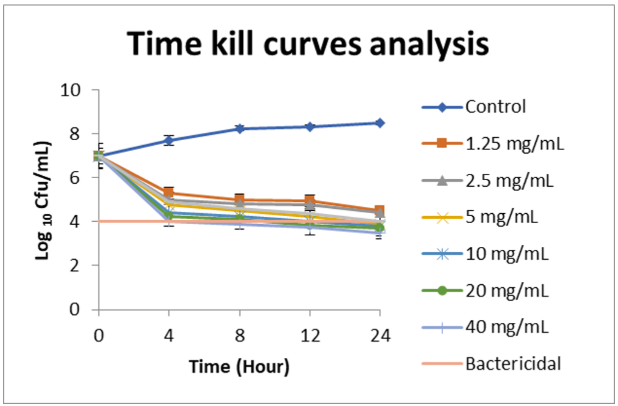

Figure 1: Time kill curves analysis of S. mutans (ATCC 25175) strains treated with MMSBAE for 24 hours at double serial dilution concentrations.

Growth profile of *S. mutans* exposed to MMSBAE.

MBC value can be subjected to technical difference and theoretical limitations error (Hanafiah *et al.*, 2015). According to the Clinical and Laboratory Standard Institute (CLSI), bactericidal activity is defined if the culture reduces more than three $\log_{10}$ CFU/mL from the initial inoculum. Whereas, bacteriostatic activity is defined if the culture reduces less than 3 $\log_{10}$ CFU/ mL from the initial inoculum (CLSI, 1999). Hence, the time-kill analysis had been done towards *S. mutans* treated with MMSBAE at these concentrations (1.25 – 40 mg/mL) to confirm the bactericidal activity of MMSBAE further while non treated *S. mutans* was used as a negative control.

The analysis showed the growth of non-treated *S. mutans* was increased every 4 hours while the growth of treated *S. mutans* was decreased for every concentration range from 10 to 40 mg/mL. MMSBAE (1×MIC and 2×MIC) reduced 2 $\log_{10}$ CFU/mL of bacteria from the initial inoculum after 4 hours treatment, and MMSBAE (16×MIC and 32×MIC) killed 3 $\log_{10}$ CFU/mL of *S. mutans* from the initial inoculum after 4 hours of treatment. MMSBAE possess bacteriostatic properties against *S. mutans* at 1×MIC and 2×MIC value. Bactericidal properties of MMSBAE was shown at 4×MIC, 8×MIC, 16×MIC and 32×MIC concentrations. Therefore, based on this Figure 1 growth curve, MMSBAE showed the characteristics of a concentration-dependent bacteriostatic agent. This result contradicted with MIC, and MBC assay, which reported at 1×MIC MMSBAE was presumed as a bactericidal agent.

Anti-biofilm activity of MMSBAE

Reduction pattern in biofilm formation was concentration-dependent manner (Table 2). More concentration was reducing more biofilm formation on *S. mutans*. About 50% of biofilm formation was inhibited at 1.25 mg/mL (1×MIC). At MBC (5 mg/mL) value, biofilm formation was reduced to 46.1%. Meanwhile, NaF (1 mg/mL) reduced biofilm formation to 16%.

The results indicate that higher concentration comprised more bioactive compounds as compared to lower MMSBAE concentration. Previous studies reported that high concentration extracts increased their anti-bacterial activity (Hanafiah *et al.*, 2015; Yoshida and Kuramitsu, 2002). This proved that extract concentration is an essential element in determining the anti-bacterial activity of various plants.

SEM and TEM analyses

SEM analysis in Figure 2 showed *S. mutans* cells morphology that is in coccus (C) was altered after treated with 1×MIC MMSBAE at 4 and 8 hours. At 4 hours, a few *S. mutans* cells were found shrunk (S) and lysed (L) meanwhile, and other *S. mutans* cells remained in a chain formation. At 8 hours, most of the bacterial cells were shrunk and lysed when compared to non-treated bacterial cells which were coccus in shape and chain in a cluster. This result is in concordance with time-kill assay analysis, which reported *S. mutans* growth was reduced to <3 $\log_{10}$ CFU/ mL at 4 hours after exposed with 1×MIC MMSBAE.

TEM analysis in Figure 3 showed cell membrane loss and cell shape was changed from coccus (C) to elongated (E) form at 4 hours of treatment with 1×MIC MMSBAE. Some of the cytoplasms were expelled out when compared to non-treated bacterial cells. Morphology of non-treated *S. mutans* cells was coccus (C), rigid cell membrane (M), high cytoplasm content with periplasmic space. At 8 hours of treatment, most of the bacterial cells lost cell membrane (L)and caused the cytoplasm expelled out.

SEM and TEM analyses revealed that there were changes observed on *S. mutans* cell morphology such as ruptured cell membrane and loss of cytoplasm content after exposed with 1×MIC MMSBAE. Most anti-bacterial agents penetrate through cell membrane then affect the targeted area. The previous study suggested that bioactive compounds from plant extract affected lipids hydrophobic bonds in cell membranes. This hydrophobic interaction is an important parameter to observe cell membrane integrity. This study suggested that hydrophobic interaction between MMSBAE organic groups and lipid from cell membrane caused changes in *S. mutans* cell morphology. The previous study on

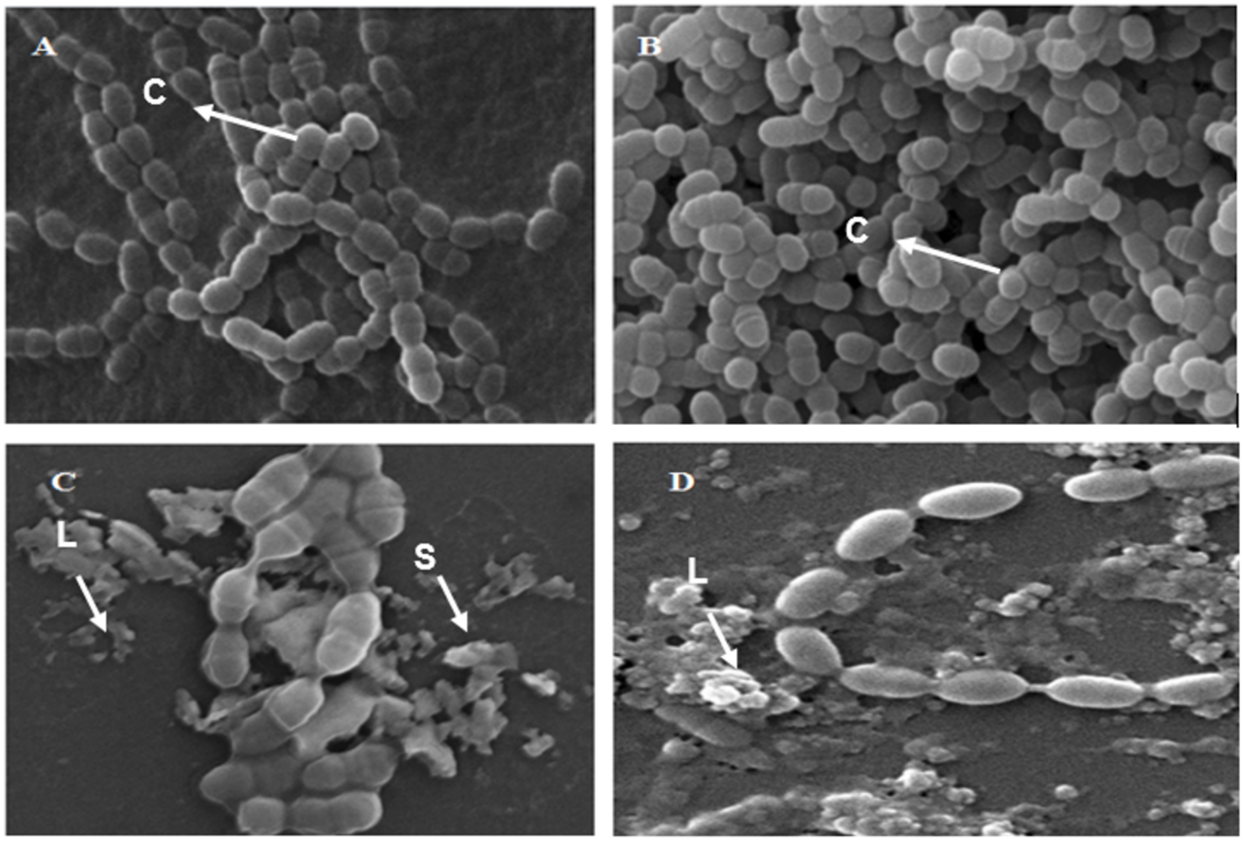

Figure 2: SEM micrograph (20000 × magnification) of A) Non-treated S. mutans at 4 hours, B) Non-treated S. mutans at 8 hours, C) S. mutans treated with 1xMIC MMSBAE at 4hours and D) S. mutans treated with MMSBAE at 8 hours.

GCMS analysis reported MMSBAE possessed ethyl ester and gamma sitosterol compounds (Hanafiah *et al.*, 2015). Ethyl ester is a functional group that found in many bioactive compounds with anti-bacterial properties. Whereas gamma sitosterol exhibited anti-bacterial and antifungal properties (Hanafiah *et al.*, 2015, 2018).

Effects of MMSBAE on *S. mutans* gene expression.

Two samples of RNA were sequenced for this study. Those were *S. mutans* treated with 1×MIC MMSBAE (1.25 mg/mL) and non-treated *S. mutans* (control). RNA integrity number (RIN) for *S. mutans* treated and non-treated with MMSBAE were 8.8, respectively. Total RNA is considered good quality if the RIN is >5 and deemed perfect if >8 (Fleige and Pfaffl, 2006).

The reference gene that has been chosen for relative gene expression analysis was 16S rRNA gene. Reference gene is important to stabilize its constant expression in all environmental condition. The reference gene is measured stable if standard deviation values < 1; meanwhile, the reference gene is unstable if the standard deviation values > 1 (Fleige and Pfaffl, 2006). PCR efficiency of 16S rRNA and r^2 were 95.3 and 0.972, respectively. These standards values were followed the requirement in minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (Bustin *et al.*, 2009).

Transcriptome analysis showed an enormous number of genes regulation in *S. mutans* that treated with 1×MIC MMSBAE. A total of 1721 genes have been successfully aligned with *S. mutans* NN2025, whereby 926 genes were upregulated, and 795 genes were downregulated (Table 3). Data selected was based on p<0.05 value. Fold change values had been selected between less than -0.05 and more than 0.05 (Adnan *et al.*, 2017). Gene regulation of *S. mutants* in response to MMSBAE involved several pathways including RNA synthesis, protein synthesis and lipid metabolism. However, this study focused on biofilm formation, glycolysis, Krebs cycle and peptidoglycan biosynthesis pathways.

NGS analysis revealed genes involved in glycolysis, biofilm formation, Kreb cycle and peptidoglycan biosynthesis pathways were downregulated when *S. mutans* treated with 1×MIC MMSBAE.

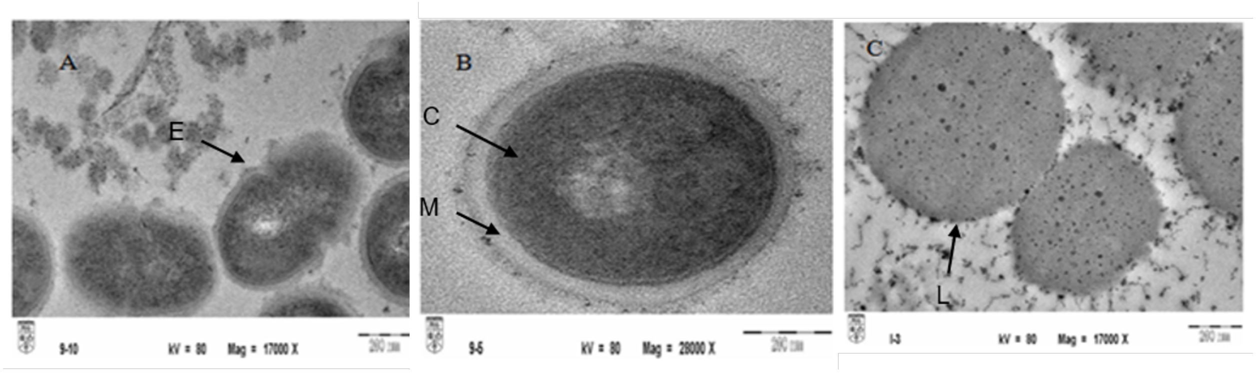

Figure 3: TEM micrograph (17K-20K × magnification) of A) S. mutans treated with MMSBAE at 4 hours, B) Non-treated S. mutans at 8hours and C) S. mutans treated with MMSBAE at 8 hours.

Table 4 showed differential expression regulation of genes that involved in glycolysis, biofilm formation, Kreb cycle and peptidoglycan biosynthesis pathways when *S. mutans* treated with 1×MIC MMSBAE for 4 hours.

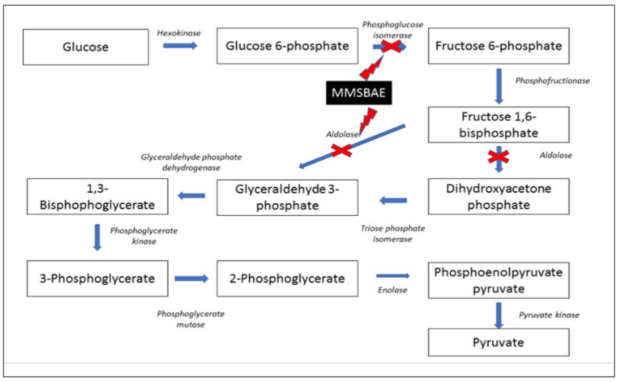

Figure 4: An overview of MMSBAE inhibits enzyme phosphoglucose isomerase (Pgi) and aldolase, causing inhibition of pyruvate production.

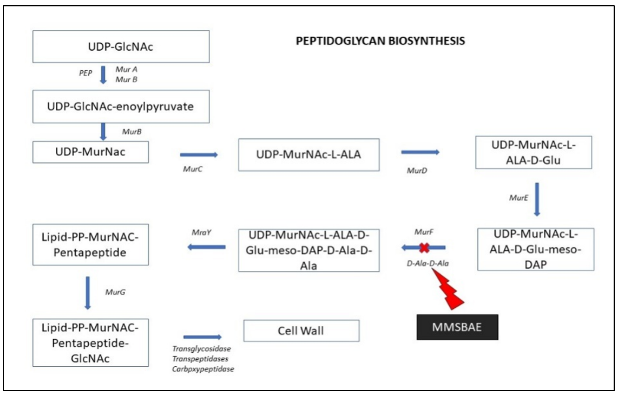

Figure 5: Pathway of peptidoglycan biosynthesis after treated with MMSBAE, which eventually inhibit the peptidoglycan biosynthesis.

Genes involved in biofilm formation such as *gtfA* (-3.00), *gtfB* (-0.43), *gtfC*(-0.95) and *gtfD* (-0.27) were downregulated when *S. mutans* treated with 1×MIC MMSBAE. Downregulated of these genes will prevent biofilm formation activity of *S. mutans* on the tooth surface. Those genes are responsible for establishing glycosidic linkages. The genes were catalyzed saccharide moieties transfer and produced polymer glucan from carbohydrates (Bowen *et al.*, 1991). Polymer glucan is a vital substrate to bind with glucan binding protein (gbp). The proteins were produced by *gbp* gene. This analysis showed *gbpA* gene was downregulated at -0.88 when *S. mutans* were treated with 1×MIC MMSBAE. Glucan binding protein is used to bind with glucan polymer for the energy production process. Lack of this gene will affect the biofilm process. B*rpA* gene was downregulated (-0.41) when *S. mutans* treated with 1×MIC MMSBAE. Inhibition of this gene will disable *S. mutans* attachment ability on the tooth surface (Yoshida and Kuramitsu, 2002). These results were in concordance with SEM analysis whereby *S. mutans* were shrunk and separated from each other at 1×MIC MMSBAE.

NGS analysis showed quorum sensing genes were downregulated such as *comD*, *comEB*, *Lux*s, and *her* genes when *S. mutans* treated with 1×MIC MMSBAE. Differential gene expression regulation of those genes were -0.08 (*com*D), -0.63 (*com*EB), *Luxs* (-0.04), and *hpr* (-3.405). Phosphoglucose isomerase (*Pgi*) gene was downregulated (-2.9843) when *S. mutans* treated with 1×MIC MMSBAE. The function of this gene is to convert 6-glucose -phosphate to 6-fructose-phosphate in glycolysis pathway (Figure 4) (whan Kim and Dang, 2005). This analysis also revealed genes involved in cell division were downregulated when *S. mutans* treated with MMSBAE. Genes *fts*Z and *fts*A genes were downregulated when compared with non-treated *S. mutans* at -1.919 and 0.4236, respectively. This result is in concordance with Monahan et al. (2014) which reported bacteria division activity would be interrupted if glycolysis activity is disrupted (Monahan

Table 1: Sequence of primers in Real-Time PCR analysis

Genes	Primers
Phosphoglucose isomerase	(F) AGCTGCAAGAGATAAGGCCA (R) AAACTACCCCCGATGCCTAC
D-alanine-D-alanine ligase	(F) CCAAATGGCGTACTGTTGTG (R) AAAACCTCAGCCCAAACCTT
Glucan binding protein A	(F) ATGGCGGTTATGGACACGTT (R) TTTGGCCACCTTGAACACCT
Transcriptional regulator	(F) GGAGGAGCTGCATCAGGATTC (R) AACTCCAGCACATCCAGCAAG
Deoxycytidylate deaminase	(F) ACGGGTTACAATGGTGGTGT (R) GACGCCTGCCTGTAAGAGAG
Histidine kinase	(F) TTGTCCCTATAGCCCCTGAA (R) GGAGGAACAGCCCATTGTAA
Asparaginyl-tRNA synthase	(F) TTTGGTGAGCCTGCTTTTCT (R) TCTGCATCCATCATCCAAAA
Protein ribosome	(F) AGTGGTCGTGATGCATTAGAAG (R) GCTCTGGACGAACTTCTACTG
16S rRNA	(F) CCTACGGGAGGCAGCAGTAG (R) CAACAGAGCTTTACGATCCGAAA

Table 2: Biofilm percentage of treated *S. mutans* with MMSBAE (0-40 mg/mL) and NaF (1mg/mL).

Concentration of MMSBAE (mg/mL)	0	0.3125	0.625	1.25	2.5	5	10	20	40	NaF
Biofilm Percentage %	100.00 ±0.05	57.00 ±0.02	55.00 ±0.01	50.00 ±0.03	48.00 ±0.06	46.00 ±0.05	42.00 ±0.08	29.00 ±0.1	25.00 ±0.08	16.00 ±0.01

Table 3: Total of genes successfully mapped and compared with *S. mutans* NN2025. The analysis showed 926 genes were upregulated. Meanwhile, 795 genes downregulated.

Sample	Overall Genes		
	Total	Upregulation (Treated gene > Untreated gene)	Upregulation (Treated gene < Untreated gene)
RNA	1721	926	795

et al., 2014). On the other hand, D-alanine-D alanine (*ligase*) gene was suppressed at -0.1032 when *S. mutans* treated with 1×MIC MMSBAE. This gene is responsible for producing enzyme ligase, as illustrated in Figure 5 (Bruning *et al.*, 2011). Lack of this gene caused difficulty for *S. mutans* to produce peptidoglycan after exposed with 1×MIC MMSBAE.

Several downregulated genes from NGS analysis had been chosen to validate with RT-PCR. RT-PCR analysis showed gene expression level was decreased and in concordance with NGS analysis. *Pgi*, *ligase*, *gbp*A and other genes were downregulated when *S. mutans* treated with 1× MIC MMSBAE. *Pgi*genes involved in glycolysis pathway while ligase is responsible in the biosynthesis of peptidoglycan. Meanwhile. *gbp*A, *brp*A, *ComEB*and *CovS* genes are responsible for the formation of biofilm and quorum sensing process. *AsnS* gene is responsible for aminoacyl-tRNA synthesis (Cassels *et al.*, 1995). On the other hand, *rpSC* gene is involved in protein ribosome synthesis (Maguire, 2009). All of these genes were downregulated in NGS analysis and showed the same pattern after validation with RT-PCR analysis (Table 5).

Table 4: Differential gene expression regulation of *S. mutans* treated with MMSBAE. The differential regulated gene had been done by comparing to non-treated bacteria.

Gene	Product	Relative gene expression (Fold change)
gtfA	Sucrose phosphorylase	-3.00
gtfB	Glucosyltransferase-I	-0.43
gtfC	Glucosyltransferase-SI	-0.95
gtfD	Glucosyltransferase-S	-0.27
gbpA	Glucan-binding protein A	-0.88
brpA	Transcriptional regulator	-0.41
comD	Histidine kinase	-0.08
comEB	Deoxycytidylate deaminase	-0.63
Luxs,	Autoinducer-2 production protein	-0.04
hpr	Serine kinase	-3.405
Pgi	Phosphoglucose isomerase	-2.9843
ftsZ	FtsZ protein	-1.9190
ftsA	FtsA protein	-0.4236
Cov	Histidine kinase	-1.0500
asn	Asparaginyl-tRNA synthase	-1.0900
rpSC	Protein ribosome	-2.9543
ligase	D-alanine-D-alanine ligase	-0.1032

Table 5: Gene expression level from RT-PCR analysis when *S. mutans* treated with MMSBAE.

Genes	Protein encode	Gene expression level	
		RT-PCR (Fold change)	NGS (Fold change)
Pgi	Phosphoglucose Isomerase	-1.735	-2.9843
Ligase	D-Alanine-D-Alanine Ligase	-0.245	-0.1032
gbpA	Glucan-binding protein A	-0.562	-0.8800
brpA	Transcriptional regulator	-0.161	-0.4100
ComEB	Deoxycytidylate deaminase	-0.523	-0.6300
CovS	Histidine kinase	-0.186	-1.0500
asnS	Asparaginyl-tRNA synthase	-0.197	-1.0900
rpSC	Protein ribosome	-0.165	-2.9543

NGS analysis showed MMSBAE disturbed the cell biology process, especially in quorum sensing, glycolysis, Kreb cycle and peptidoglycan formation. Differential gene expression of quorum sensing such as *ComD*, *ComEB*and *Luxs* was downregulated when *S. mutans* treated with MMSBAE. This result is in concordance with SEM and TEM analyses which showed *S. mutans* cell morphology was changed and lysed at 1× MIC MMSBAE after 4 hours of treatment. Lack of these genes will cause disruption in quorum sensing as well as biofilm-forming activity. Transcriptome analysis also showed *the hpr* gene was downregulated when *S. mutans* treated with MMSBAE. This gene encodes a kinase enzyme which is responsible for producing enzyme and protein that involved in glucose transportation (Moye *et al.*, 2014). *Hpr* gene is also responsible for producing serine kinase protein which is important to induce production of the substrate in the glycolysis pathway. Inhibition of *the hpr* gene will disturb the production of pyruvate in the glycolysis pathway.

Pyruvate is an important substrate to produce energy in the glycolysis pathway. MMSBAE mechanism of action which disturbed the glycolysis pathway is a novel anti-bacterial agent by preventing the formation of *S. mutans* biofilm. Based on NGS analysis, cell division genes such as *ftsZ* and *ftsA* were downregulated when *S. mutans* treated with MMSBAE. This analysis was in concordance with the study done by Monahan et al. (2014)

which reported disruption of glycolysis pathway decreased cell division activity of bacteria (Monahan *et al.*, 2014).

This study also revealed the mechanism of action of MMSBAE towards peptidoglycan biosynthesis pathway. MMSBAE suppressed genes that encode enzyme D-alanine-D-alanine ligase. This resulted in UDP-MurNAc-L-ALA-D-Glu-Meso-DAP substrate were not lysed to UDP- MurNAc-L-ALA-D-Glu-Meso-DAP-D-Ala-D-Alasubstrate and eventually caused inhibition in peptidoglycan synthesis. Peptidoglycan synthesis pathway is important in structuring bacterial cell membrane. This finding is in concordance with SEM and TEM analyses which found that *S. mutans* have lost cell membrane contents when the bacteria treated with 1× MIC MMSBAE. John et al. (2011) reported that peptidoglycan synthesis in *Mycobacterium tuberculosis* could not be produced due to downregulated of enzyme D-alanine-D-alanine ligase. They also suggested this enzyme is targeted to inhibit the growth of bacteria (Bruning *et al.*, 2011).

Real-time PCR analysis showed that several genes such as *Pgi, Ligase, gbpA, brpA, ComEB, CovS, asnS and rpSC*were downregulated when *S. mutans* treated with 1× MIC MMSBAE. The findings validated the results from NGS analysis which showed all mentioned genes were downregulated when treated with 1× MIC MMSBAE.

CONCLUSIONS

Overall, the data from this study has successfully highlighted the potential of MMSBAE as an antibacterial agent against *S. mutans*.

ACKNOWLEDGEMENT

Malaysian Genomic Research centre for NGS service and Malaysian Genome Institute for bioinformatics training.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

Funding support

Universiti sains Islam Malaysia financially supported this study (P1-16-17019-UNI-USIM-FPG) and Ministry of High Education (MOHE) Malaysia (USIM/FRGS/FPG/055002/51717).

REFERENCES

Adnan, S.-N.-A., Ibrahim, N., Yaacob, W. A. 2017. Transcriptome analysis of methicillin-resistant Staphylococcus aureus in response to stigmasterol and lupeol. *Journal of Global Antimicrobial Resistance*, 8:48–54.

Bowen, W. H., Schilling, K., Giertsen, E., Pearson, S., Lee, S. F., Bleiweis, A., Beeman, D. 1991. Role of a cell surface-associated protein in adherence and dental caries. *Infection and Immunity*, 59(12):4606–4609.

Bruning, J. B., Murillo, A. C., Chacon, O., Barletta, R. G., Sacchettini, J. C. 2011. Structure of theMycobacterium tuberculosisd-Alanine:d-Alanine Ligase, a Target of the Antituberculosis Drug d-Cycloserine. *Antimicrobial Agents and Chemotherapy*, 55(1):291–301.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., Wittwer, C. T. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, 55(4):611–622.

Cassels, R., Oliva, B., Knowles, D. 1995. The occurrence of the regulatory nucleotides ppGpp and pppGpp following induction of the stringent response in staphylococci. *Journal of bacteriology*, 177(17):5161–5165.

CLSI 1999. Methods for determining bactericidal activity of antimicrobial agents: approved guideline M26-A. *Clin. Lab. Stand. Inst*, 19(7).

Fleige, S., Pfaffl, M. W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*, 27(2-3):126–139.

Hanafiah, R. M., Aqma, W. S., Yaacob, W. A., Said, Z., Ibrahim, N. 2015. Antibacterial and biofilm inhibition activities of Melastoma malabathricum stem bark extract against Streptococcus mutants. *Malaysian Journal of Microbiology*, 11(2):199–206.

Hanafiah, R. M., Ghafar, S. A. A., Ibrahim, Z., Ahmad, W. Y. W., Noor, W. S. A. W. M., Ibrahim, N. 2018. Anti-Bacterial Activities of Melastoma Malabathricum Stem Bark Fractions against Streptococcus Mutans. *Malaysian Journal of Science Health and Technology*, (1):52–56.

Javed, A., Chaudhry, S., Butt, S., Ijaz, S., Asad, R., Awais, F., Khan, A. A. 2012. Transmission of streptococcus mutans from mother to child. *Pakistan Oral and Dental Journal*, 32(3):493–496.

Joffrey, S. M., Yob, N. J., Rofiee, M. S., Affandi, M. M. R., Suhaili, Z., Othman, F., Zakaria, A, Z. 2012. Melastoma malabathricum (L.) smith ethnomedicinal uses, chemical constituents, and pharmacological properties: a review. *Evidence-Based Complementary and Alternative Medicine*.

Lee, D. H., Seo, B. R., Kim, H. Y., Gum, G. C., Yu, H. H., You, H. K., You, O, Y. 2011. Inhibitory effect of Aralia continental on the cariogenic properties of Streptococcus mutans. *Journal of Ethnopharmacology*, 137(2):979–984.

Liao, Y., Brandt, B. W., Li, J., Crielaard, W., Loveren, C. V., Deng, D. M. 2017. Fluoride resistance in Streptococcus mutans: a mini review. *Journal of Oral Microbiology*, 9(1):1344509–1344509.

Livak, K. J., Schmittgen, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2-\Delta\Delta CT$ Method. *Methods*, 25(4):402–408.

Maguire, B. A. 2009. Inhibition of Bacterial Ribosome Assembly: a Suitable Drug Target? *Microbiology and Molecular Biology Reviews*, 73(1):22–35.

Maji, S., Dandapat, P., Ojha, D., Maity, C., Halder, S. K., Mohapatraâ, P. K. D., Mondal, C, K. 2010. In vitro antimicrobial potentialities of different solvent extracts of ethnomedicinal plants against clinically isolated human pathogens. *Journal of Phytology*, 2(4):57–64.

Monahan, L. G., Hajduk, I. V., Blaber, S. P., Charles, I. G., Harry, E. J. 2014. Coordinating Bacterial Cell Division with Nutrient Availability: a Role for Glycolysis. *mBio*, 5(3):1–13.

Moye, Z. D., Zeng, L., Burne, R. A. 2014. Fueling the caries process: carbohydrate metabolism and gene regulation by Streptococcus mutans. *Journal of Oral Microbiology*, 6(1):24878.

Shai, L. J., McGaw, L. J., Aderogba, M. A., Mdee, L. K., Eloff, J. N. 2008. Four pentacyclic triterpenoids with antifungal and antibacterial activity from Curtisia dentata (Burm.f) C.A. Sm. leaves. *Journal of Ethnopharmacology*, 119(2):238–244.

Susanti, D., Sirat, H. M., Ahmad, F., Ali, R. M., Aimi, N., Kitajima, M. 2007. Antioxidant and cytotoxic flavonoids from the flowers of Melastoma malabathricum L. *Food Chemistry*, 103(3):710–716.

whan Kim, J., Dang, C. V. 2005. Multifaceted roles of glycolytic enzymes. *Trends in Biochemical Sciences*, 30(3):142–150.

Yoshida, A., Kuramitsu, H. K. 2002. Multiple Streptococcus mutans Genes Are Involved in Biofilm Formation. *Applied and Environmental Microbiology*, 68(12):6283–6291.

Zakaria, Z. A., Nor, R. R. M., Kumar, G. H., Ghani, Z. A., Sulaiman, M. R., Devi, G. R., Jais, A. M., Somchit, M. N., Fatimah, C. A. 2006. Antinociceptive, anti-inflammatory and antipyretic properties of Melastoma malabathricum leaves aqueous extract in experimental animals. *Canadian Journal of Physiology and Pharmacology*, 84(12):1291–1299.