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Antibacterial and antibiofilm analyses of *Melastoma malabathricum* leaves extract against *Streptococcus mutans* on tooth surfaces

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ABSTRACT

Aims: Dental caries is a chronic infectious disease caused by *Streptococcus mutans* due to its ability to form biofilm. This study aims to assess the antimicrobial efficacy of *Melastoma malabathricum* leaf extract against *S. mutans* on the surface of tooth samples as a potential therapy for dental caries.
Methodology and results: Extraction of *M. malabathricum* leaves was done using acetone as the solvent and antibacterial activity of the extracts was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Antibiofilm activity of *M. malabathricum* extract against *S. mutans* was determined by comparing the colony count, biofilm formation assay and morphology observation by scanning electron microscope (SEM). The MIC value of extracts was 6.25 mg/mL and MBC value was >25 mg/mL. A decrease in colony count was noted when tooth samples were incubated with *M. malabathricum* extract for 8 h compared to 4 h incubation. At pH 5, the formation of the colony was the least, medium at pH 8 and maximum at pH 7. A decrease in biofilm formation was observed when tooth samples were incubated with the extract for 8 h. SEM observations showed treatment with the extract caused *S. mutans* cell membrane to leak leading to cell morphology changes.
Conclusion, significance and impact of study: Acetone extract of *M. malabathricum* leaves showed excellent antibacterial activity against *S. mutans*. It has bactericidal activity with the ability to inhibit biofilm in dose-dependent manner against *S. mutans*. The morphological analyses suggested that the extract disrupted the cell membrane of the bacteria.

Keywords: antibacterial, biofilm, *Melastoma malabathricum*, *Streptococcus mutans*

INTRODUCTION

Dental caries or cavities is classified as a common chronic infectious disease caused by bacteria that demineralises the surface of the tooth. The disease can progress into severe pain, bacteraemia and eventually tooth loss. Report from National Health and Nutrition Examination Survey noted that 42% of children aged two to eleven have had dental caries in their primary teeth and 92% will experience tooth decay during their adulthood (Dye *et al.*, 2007). This problem increased in severity when antibiotics such as penicillin, cephalosporin and tetracycline that were commonly used to treat mouth infection become less effective in treating pathogenic microorganisms (Bidault *et al.*, 2007). Misuse and overuse of antibiotics are also contributing factors to the increase in bacterial resistance. In addition, the common mouthwash available to prevent dental decay contains high levels of alcohol that may lead to side effects such as mouth cancer (Lachenmeier, 2008).

Bacteria that cause dental caries are able to increase acid production from carbohydrates such as sucrose, fructose and glucose (Karpinski and Szkaradkiewicz, 2013). The level of acid formed in the mouth will affect the teeth due to the special mineral content found within the teeth that is sensitive to low pH. *Streptococcus mutans* is the main bacteria found in the mouth that cause tooth cavity and biofilm formation (Yang *et al.*, 2012). In the presence of extracellular glucose and sucrose, *S. mutans* is able to synthesise polysaccharide such as glycogen (Peterson *et al.*, 2011) and also produce mutagens such as bacteriocins which are considered to be an important factor in the formation of colonies and biofilm formation on the teeth surface (Merritt and Qi, 2012). Therefore, it is necessary to develop novel agents which can selectively inhibit pathogenic bacteria and biofilms. According to

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Bidault *et al.* (2007), the search for a new dental caries treatment is prudent since the current available treatment showed limited results. Previously, Rohazila *et al*. (2015) have shown antibacterial and antibiofilm activities of *Melastoma malabathricum* stem bark extract on *S. mutans* by employing biofilm formation assay in 96-well plate. *M. malabathricum* or also known as rhododendron is a herbal plant in the Melastomatacae family. It is found in the tropical and subtropical regions from all over the world and consists of 4000 different species. The presence of flavonoid, triterpene, saponins and steroids in *M. malabathricum* contributes to the ability of the plant to demonstrate antibacterial (Alwash *et al.*, 2013; Rohazila *et al*., 2015), antiviral (Nazlina *et al.*, 2008), antioxidant (Alwash *et al.*, 2014), antiparasitic, antiulcer, anti-inflammatory as well as wound healing properties (Zakaria *et al.*, 2006; Joffry *et al.*, 2012). In this study, the antibacterial and antibiofilm activities of *M. malabathricum* leaf acetone extract against *S. mutans* on tooth samples were determined at different pHs using a microtitre plate format. The current research enable us to directly determine the antibacterial and antibiofilm activities of the leaf extract towards tooth samples.

MATERIALS AND METHODS

Extract preparation

Melastoma malabatricum leaf was collected near the Universiti Kebangsaan Malaysia greenhouse (2°55'39.8" N101, 46'33.0"E). Extraction was done with modification according to Rohazila *et al.* (2015). *Melastoma malabathricum* leaf powder (100 g) was soaked overnight in 500 mL of acetone at room temperature. The extract was then filtered through Whatman No.1 filter paper and evaporated using Heidolph rotary evaporator at 40 °C. The concentrated extract was in liquid form and stock concentration was prepared as 25 mg/mL in 10% (v/v) dimethyl sulfoxide (DMSO).

Bacterial strain

The bacteria used in this experiment was *Streptococcus mutans* (ATCC 25175) cultured on Brain Heart infusion (BHI) agar (Oxoid) and incubated at 37 °C for 18 h in an anaerobic jar. The culture was inoculated into BHI broth (Oxoid) and incubated at 37 °C in anaerobic conditions for 24 h.

Tooth samples

Samples were provided by the Faculty of Dentistry, Islamic Science University of Malaysia from discarded tooth from aesthetic patients which was randomly selected and in good condition. The tooth samples were brushed and sterilised by autoclaving (121 °C, 15 min). Samples were placed in phosphate buffer saline (PBS, pH 7.4) and stored at room temperature. To prevent formation of biofilm during storage, PBS was replaced weekly by using fresh sterile PBS until the antibacterial and antibiofilm activity assay were conducted.

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained using two-fold serial dilution according to the Alwash *et al.* (2013) method with some modifications. The MIC was done in a sterile round bottom 96-well microplate. Pure extract with concentration of 25 mg/mL was added in the first well. The following well containing 100 µL of Mueller Hinton Broth (MHB) (Oxoid) with varying concentrations of *M. malabathricum* extract (0.39-12.5 mg/mL). Following that, 100 µL of overnight *S. mutans* culture (10^8 CFU/mL) was added to each well to obtain the final volume of 200 µL per well. DMSO (10%) with a volume of 200 µL was used as negative control. Chlorhexidine (Sigma) (200 µL) at 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 mg/mL was used as positive control. All tests were performed in duplicates. The plates were incubated for 24 h at 37 °C in anaerobic condition.

After incubation, 25 µL of 3-(4,5-dimethylthiazo-2-il)-2,5-diphenyltetrazolium bromide (MTT) was added in to each well and was incubated for another two hours. Colour change from yellow to blue indicates the formation of formazan by living cells. The MIC value was determined as the lowest concentration that inhibits the visible growth of bacteria based on the MTT assay. To determine the minimum bactericidal concentration (MBC), 5 µL of solution was aliquoted from the MIC well with no bacterial growth and was spread on nutrient agar (NA) (Oxoid) and incubated overnight at 37 °C. Colony growth was observed after 24 h incubation. The MBC was defined as the lowest concentration preventing bacterial growth. Each assay was performed with duplicate samples and was repeated three times.

Biofilm formation on tooth surface

The assay was done according to Subbiya *et al.* (2013) method with some modifications. A single colony of *S. mutans* was inoculated into MHB with pH 5, 7 and 8 that has been adjusted with HCl and NaOH. The *S. mutans* culture was also inoculated into MHB with pH 7.4 as control. The inoculated *S. mutans* were then incubated at 37 °C for 24 h in anaerobic conditions. Next, *S. mutans* (1.5 mL of OD 0.5 McFarland standard (1.5×10^8 CFU/mL) was added to each well of 24-well plate containing tooth samples (triplicate for each treatment) and covered with parafilms. The plate was incubated at 37 °C for 24 h in anaerobic conditions. After incubation, all tooth samples were transferred to a new 24-well plate and 1.5 mL of *M. malabathricum* extract with a concentration acquired from MIC was added to each well except the control. The plate was then incubated for 24 h at 37 °C for 4 h and 8 h. The tooth samples with the *S. mutans* biofilms were then used for antibacterial activity and biofilm assay.

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Antibacterial analysis

After incubation with extract, antibacterial analysis was performed according to Subbiya *et al.* (2013) method. The analysis was performed by firstly transferring the tooth sample into small Universal bottles containing 1 mL of MHB at varying pH and the solution was slowly vortex. Following that, serial dilution was performed and 100 µL of the solution (10^{-4} and 10^{-6}) was taken and spread on NA plates. The plates were then incubated at 37 °C for 24 h in anaerobic conditions. Plate count method was done in duplicate for each dilution. Colony Forming Unit (CFU/mL) was counted according to the number of colonies formed on each plate. Analysis was performed with triplicate samples and was repeated three times.

Biofilm formation assay

The biofilm formation assay was performed in accordance to Rohazila *et al.* (2015) method. After the tooth sample (triplicate for each treatment) was soaked in the leaf extract for 4 h and 8 h, the medium was removed and the tooth sample was washed with PBS twice. Sample was stained with 1 mL of 1% (w/v) of crystal violet for 15 min and rinsed with distilled water. Then, the tooth sample was transferred to a small universal bottle containing 1 mL of 99% ethanol and the solution was vortex slowly. The turbidity of the solution was measured using the spectrophotometer at 595 nm wavelength to measure the biofilm formation. Analysis was performed with triplicate samples and was repeated three times.

Scanning electroscope microscope analysis (SEM analysis)

Following the incubation with *M. malabathricum* extract, *S. mutans* on the surface of the tooth were fixed with 2% (v/v) glutaraldehyde. The tooth samples were then incubated at 4 °C for 24 h. After fixation, the samples were rinsed with 0.1 M of PBS. The Critical Point Drying method (Bray, 2000) was performed for the SEM analysis whereby samples were firstly dried in a gradual manner with the slow increment of ethanol which acts as a dehydrating agent. The samples were dried until critical point is reached in liquid carbon dioxide which aids as a transitional solvent. The samples were then attached to special plates known as stubs, coated in a thin layer of gold and was observed under the SEM.

Statistical analysis

All experiments were carried out in triplicates in at least three different occasions. The differences between the two means were evaluated by the Student's t-test. The data were analysed by one-way ANOVA for comparison of multiple means. The level of significance for all statistical tests was set at $p<0.05$.

RESULTS AND DISCUSSION

Melastoma malabathricum leaf extraction

Table 1 shows the results of the percentage of *M. malabathricum* acetone extraction. The *M. malabathricum* leaves extract by using acetone yielded 1.03% (w/w). Acetone is among of organic solvent which commonly used to extract antibacterial compounds from plants (Rathish and Sumitra, 2007). Studies have shown that plant extracts produced using acetone as an extraction agent is known to have excellent antimicrobial properties (Gami and Parabia, 2011). Ashok *et al.* (2014) have revealed the treatment of *Psidium guajava* extract by using acetone against *S. mutans* was more effective when compared to ethanol and methanol.

Table 1: Yield of *M. malabathricum* acetone extraction.

Weight of leaves after drying (g)	Weight of extract (g)	Yield (%)
103	1.06	1.03

Antibacterial activity

According Table 2, the MIC value of the *M. malabathricum* extract and chlorhexidine towards *S. mutans* is 6.25 mg/mL and 0.78 mg /mL. Previous studies have shown that the MIC value of ginger extract and garlic extract towards *S. mutans* is 12.5 mg/mL and 50 mg/mL respectively (Jain *et al.*, 2015). Hence, it can be deduced that the acetone extract of *M. malabathricum* has better *S. mutans* inhibiting activity as it shows the lowest MIC value when compared with ginger and garlic extract extracted using chloroform, acetone and ethanol respectively. The MIC value indicates the lowest level of antimicrobial required to inhibit the growth of the microorganism (Basri *et al.*, 2011), while MBC value indicates the minimum concentration of an antimicrobial agent required to kill most of the viable microorganisms (Alwash *et al.*, 2013). The MBC value for both *M. malabathricum* and chlorhexidine towards *S. mutans* is higher when compared to the MIC value which is >25 mg/mL. This indicates that it requires a relatively high concentration of antimicrobial to kill these bacteria.

According to French (2006), an antibacterial agent is considered to be bacteriocins if its MBC value is not four time more than the MIC value. The *M. malabathricum* leaf extract is considered to be bacteriostatic towards *S. mutans* because the obtained MBC value is higher than the tested concentration (0.39-12.5 mg/mL).

Table 2: MIC and MBC values for *M. malabathricum* extract and chlorhexidine towards *S. mutans*.

Materials	MIC value (mg/mL)	MBC value (mg/mL)
M. malabathricum extract	6.25	> 25
Chlorhexidine	0.78	> 25

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Antibacterial analysis

The antibacterial and antibiofilm tests were done as the evidence of the extended research to the previous studies done by Rohazila *et al.* (2015). Thus, this research was conducted to support the previous findings by using teeth sample in order to measure the antibacterial and antibiofilm activity of the leaf extract against bacteria. This test was done to determine the efficiency of the antibacterial activity of *M. malabathricum* extract on the *S. mutans* colony formation. Biofilm formation on the surface of the teeth was performed by incubating the teeth sample in *S. mutans* culture for 24 h (Ahn *et al.*, 2008). The extract was added after the biofilm formed and the teeth sample was incubated in the extract for two different time periods, 4 h and 8 h. However, for the control, there was no addition of extract to the teeth sample. Table 3 shows that the CFU/mL is lesser when incubated in the leaf extract for 8 h when compared to incubation of 4 h.

The number of colonies at pH 5 decreased from 8.15 × 10^9 CFU/mL to 9.45 × 10^7 CFU/mL. It is also the lowest number of colonies formed after incubation in extract when compared to others pH. On the other hand, colony formation at pH 8 is moderate for both incubations. Similarly, the number of colonies at pH 7 reduced to 2.56 × 10^8 CFU/mL from 2.01 × 10^{10} CFU/mL. However, it gives the highest number of colony formation after incubation in the extract for both 4 h and 8 h. This is because the optimum pH for *S. mutans* growth is between pH 5.5 to 7.4 which is similar to the pH of the mouth. In this study, the results show the *S. mutans* bacteria colony formation that was treated with *M. malabathricum* extract is considerably lower than the untreated bacteria. Thus, the *M. malabathricum* extract has a potential to prevent the *S. mutans* biofilm formation by inhibiting the bacteria's growth.

Biofilm formation test

The ability of natural products to inhibit the formation of biofilm is an important aspect in reducing bacterial colonisation on various surfaces. Antibiofilm agent usage is a crucial step in preventing microbial infection. Biofilm formation is an irreversible process whereby the microorganism attached itself to the surface and produces extracellular polymers that aids the attachment and matrix formation (Donlan, 2001). According to the parameters used by Culler *et al.* (2014) as reference, absorbance level at OD_{595} below 0.5 is classified as no biofilm formation. Absorbance reading below 1 indicates weak biofilm formation, while absorbance reading below 1.5 indicates moderate biofilm formation and absorbance readings that are higher than 1.5 indicates strong biofilm formation.

The absorbance level was measured at 595 nm in order to quantify the biofilm formation capacity. Overall, the biofilm formation strength reduced as the extract incubation period increases, as shown in Figure 1. The biofilm test results indicate that the *S. mutans* bacteria biofilm formation depends on the pH of the medium. At pH 5, the level of absorbance is the lowest, less than 1 which indicates poor bacterial biofilm formation. Strong biofilm formation was shown at pH 8 when compared with pH 5 and 7. The control shows the highest level of absorbance reading as there was no addition of extract to it. Attachment process in general requires nutrients, organic substance and inorganic molecules which enable

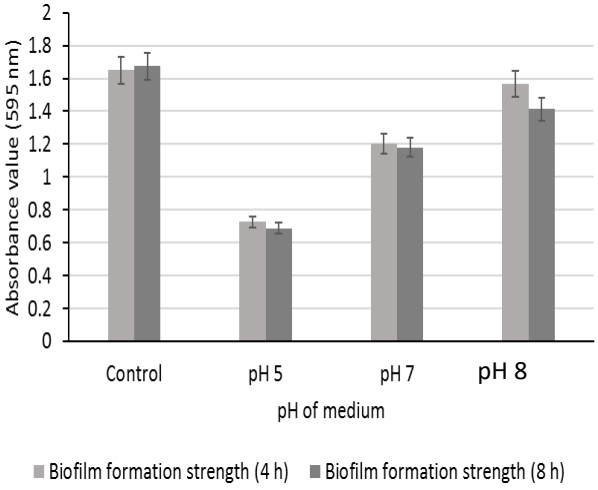

Figure 1: Absorbance value at 595 nm to measure the biofilm formation strength of *S. mutans* at pH 5, 7 and 8 after 4 h and 8 h incubation in *M. malabathricum* extract. Significant (p<0.05) reduction in biofilm activity by *S. mutans* was observed when compared to untreated cells (control).

Table 3: Total formation of bacterial colonies after 4 h and 8 h of incubation with *M. malabathricum* extract at pH 5, 7 and 8. Significant (p<0.05) reduction in antibacterial activity by *S. mutans* was observed when compared to untreated cells (control).

pH	4 h incubation (CFU/mL)		8 h incubation (CFU/mL)	
	Treated	Control	Treated	Control
5	8.15 ± 0.53 × 10^9	1.2 ± 0.02 × 10^{10}	9.45 ± 0.07 × 10^7	8.15 ± 0.05 × 10^{10}
7	2.01 ± 0.42 × 10^{10}	4.5 ± 0.32 × 10^{11}	2.56 ± 0.08 × 10^8	9.2 ± 0.06 × 10^{11}
8	1.74 ± 0.16 × 10^{10}	3.1 ± 0.22 × 10^{11}	1.91 ± 0.33 × 10^8	5.3 ± 0.1 × 10^{11}

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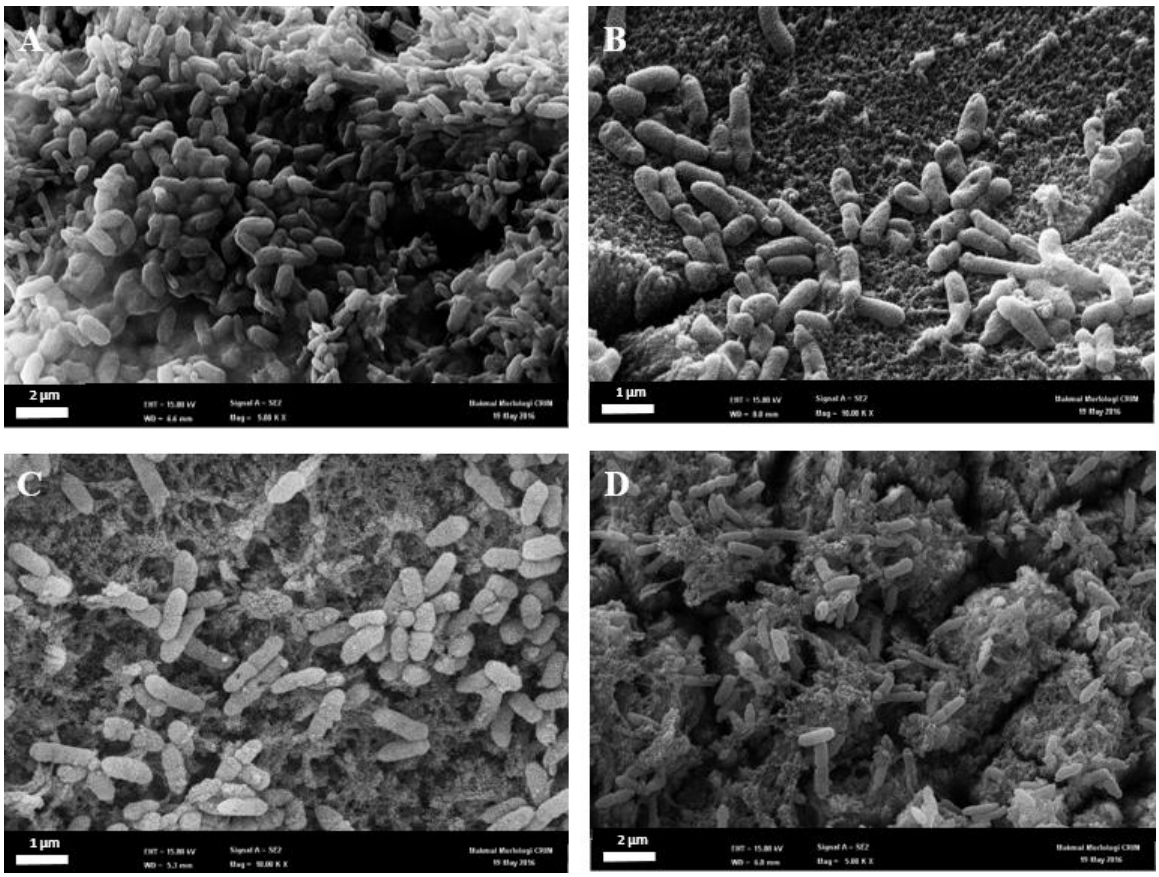

Figure 2: Scanning electron microscopy of the biofilms formed *in vitro*. (A) Untreated *S. mutans* as control under 5000 × magnification. (B-D) *S. mutans* treated with *M. malabathricum* extract incubated for 4 h at different pH. (B) pH 5 under 10000×, (C) pH 7 under 10000× and (D) pH 8 under 5000× magnification.

the attachment to occur thus forming a biofilm. Sandasi *et al.* (2010) stated that cells pre-treated with plant extract have been unable to produce a good layer which causes the cells stick and form biofilm on any surfaces. This defect could be due to the phytochemicals found in the plant extract that has a repressing effect on the bacterial growth and biofilm formation activity on the surface of the tooth. This was one of the earliest studies done on the *M. malabathricum* activity in inhibiting biofilm formation. Other plant species that have shown such inhibiting abilities includes *Polygonum cuspidatum* (Song *et al.*, 2006), *Psorolea corylifolia* (Katsura *et al.*, 2001) and *Trachyspermum ammi* (Raja *et al.*, 2011). Those studies have suggested that the inhibitory effects could be due to the presence of active compounds such as alkaloids and phenolic and such compounds were also found in the *M. malabathricum* leaf (Joffry *et al.*, 2012). Overall, *M. malabathricum* extract was able to inhibit the biofilm forming activity of *S. mutans* bacteria as reported in the studies done by Rohazila *et al.* (2015).

SEM analysis

The SEM observation results of the untreated *S. mutans* bacteria showed that the cells were clumped together and were found in layers (Figure 2A). The morphology of the cells showed round cells with smooth surface. These cells were formed in chains, clumped and in layers. The SEM micrograph of the *S. mutans* after exposure to *M. malabathricum* extract for 4 h and at pH 5 showed that the cells have changed in morphology whereby a portion of the cells have started to shrink and rupture. However, there were still cells that were in clump and chain formation. The cells in chain, however, were different in shape where they appeared to be slight elongated rather than in circular form. The cell number also have reduced, which indicates that the results obtained were in line with the antibacterial analysis tests, which showed the lowest number of bacterial count at pH 5 when compared to other pH.

The SEM observation of the *S. mutans* treated in the *M. malabathricum* for 4 h in pH 7 (Figure 2C) showed that

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the cells morphology changed slightly where a portion of the cells have expanded and were oval. There were also cells that were ruptured and shrunk. However, there were still a number of cells that appeared normal. Though the cells were still in clumps, the number of cells has reduced when compared with the control that was not exposed to the extract. Figure 2(D) shows the *S. mutans* bacterial cells that were exposed to the *M. malabathricum* extract at pH 8 for 4 h. The result of the SEM observation showed that there were cells that appeared to be ruptured, shrunken and detached from one another together with normal looking cells the cells seemed to also have changed in their morphology where they appeared to look oval in shape, rather than circular. The number of bacterial cells also has seemed to have reduced when compared with pH 7. These results are analogous to the antibacterial analysis tests, where the number of colonies at pH 8 was moderate when compared to other pH. Overall, *S. mutans* that has been treated with the *M. malabathricum* extract changes in morphology and caused the cells to detach from the chain. The SEM observation results showed that changes to the cell morphology such as, shrinking, elongating, expanding as well reducing in cell number occurs when the bacterial cells were treated with *M. malabathricum* extract when compared to cells that were not treated with the extract. These studies have shown that changes to the morphology of the bacterial cells have a direct correlation to the antibacterial analysis test.

The test deduced that that number of bacterial colonies was the lowest at pH 5, average at pH 8 and was the highest at pH 7. There was also obvious difference in terms of the morphology of the cells that were treated with the extract at pH 5 for 4 h. The results also deduced that the cells that were treated at pH 5 were much less when compared with pH 7 and 8. The changes to the morphology of the cells could be due to loss of cell content and organelles from the cytoplasm (Al-Adham *et al.*, 1998). This loss could also be the reason to the cells instability and rupture. Besides that, cell wall rupture could also be caused by interaction between the active compounds with the lipid layer of the cell wall. Straus and Hancock (2006) have reported that the compounds that were present in the plant is known to give negative effects to the lipid layer of the cell wall. In addition to that, Sofy *et al.* (2014) have suggested that the active compounds from the leaf extract can possibly attach to the surface of the cell and enter its target site which is the phospholipid layer of the cell membrane or enzyme. This could cause the cell to shrink and shrivel. However, there were some actions of the compound that causes the cells to expand and elongate in shape to an oval. This was due to the changes to the cell physiology and is uneven and causes expansion and instability of the membrane resulting in the increment to the fluid intake of the membrane.

CONCLUSION

In conclusion, the acetone extract of *M. malabathricum* leaves has good antibacterial and antibiofilm activity towards *S. mutans.* This extract is able to inhibit the growth and biofilm formation of *S. mutans*. Low concentrations of extract will suffice in inhibiting the bacterial growth, but it requires high concentration to kill the said bacteria. According to the electron microscope observations, the treatment of *M. malabathricum* extract against *S. mutans* will rupture the cell walls and cell membrane, thus causing morphological changes of the cell structure. In the antibiofilm test, the *M. malabathricum* extract was able to inhibit biofilm formation of *S. mutans* especially at low pH. However, low pH is not good for the mouth as it can cause erosion of the teeth layer that function as a protective layer and contains special minerals that makes it sensitive to low pH. Hence, further studies on the *M. malabathricum* extract are necessary to obtain active compounds that are able to kill bacteria without causing damage to the mouth cavity, as it has great potential in becoming an antibiofilm agent towards *S. mutans*.

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