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# ANTIBACTERIAL ACTIVITIES OF METHANOL EXTRACT OF *ZIZIPHUS MAURITIANA* LEAVES ON CARIOGENIC BACTERIA

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**Abstract**

Dental caries, is one of the most chronic diseases which have affected almost half of the world's population making it the major global oral health problem in the world. It is also known as biofilm-mediated multifactorial disease that occurs when the cariogenic bacteria disrupted the homeostatic balance of plaque biofilm, thus initiate disease process. Plant extracts have been used for thousands of years in the prevention and treatment of various diseases. Demand for naturally derived product that are safe, effective and economical has been increased due to adverse effect of some commercially available antibacterial agent currently used in dentistry. Thus, in the current study, methanol extracts of *Ziziphus mauritiana* (ZM) leaves have been used to study its effect on the most common cariogenic bacteria (*Streptococcus mutans* and *Streptococcus sobrinus*). The antibacterial activities were conducted using disk diffusion test (Kirby-Bauer Test). The methanol extract of ZM showed higher activity on *S. mutans* with the zone of inhibition of 15.67±0.58mm compared to *S. sobrinus* (0.00mm). The extract that showed antibacterial activity was tested to determine the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC). The MIC and MBC was observed for *S. mutans* were 100 mg/ml and 200 mg/ml respectively. Treated and untreated sample were submitted for scanning electron microscopy study. The cell treated with ZM extract showed the loss of extracellular matrix formation and disruption of the cell morphology. The phytochemicals study reveals the presence of alkaloids, phenols, tannins, flavonoids and saponin in methanol extract of ZM. Thus, this study showed the potential effect of methanol extracts from *Ziziphus mauritiana* leaves as a natural agent that can be incorporated in oral health care products.

**Keywords:** Cariogenic bacteria, *Ziziphus mauritiana*, antibacterial, cell-morphology study, Phytochemical

## 1.0 INTRODUCTION

Dental caries is a major global problem of public health and it is the most common non-communicable disease affecting humans worldwide until present years. It has the highest prevalence of all health conditions with almost half of the world's population across all age and socioeconomic status. Most of the children and adolescents are at high risk for development of dental caries (Nishikawara et al., 2007; World Health Organization (WHO), 2017). Dental caries has a complex etiology that is proposed to be activated

by three major factors which are microorganisms, environment and host (Nishikawara et al., 2007). It is the interaction between the acid producing and high tolerance towards acid environment gram positive microorganisms (cariogenic bacteria). Cariogenic bacteria will breakdown sugars that is mainly sucrose to organic acid, thus demineralizes the teeth and finally leads to dental caries (Loesche, 2007).

*Streptococcus mutans* and *Streptococcus sobrinus* are gram positive anaerobic facultative, form alpha haemolysis on blood agar and classified as mutan streptococci. Many studies reported the role of these bacteria in the initiation and progression of dental caries. Both species found to have glycosyltransferase, a key virulence factors that involves in the conversion of sucrose into lactic acid (Caufield et al., 2006; Nishikawara et al., 2007). Lactic acid produced by these cariogenic bacteria play an important role in demineralization of the tooth thus lead to dental caries. There are many factors involved in the development of dental caries. However, the presence of acid which eventually reduces the plaque pH has been reported in many studies as the main factor causing the tooth decay. Resistance of the bacteria towards antibiotics has been the major concerns for human health as it decreases the efficiency of the wide spectrum antibiotics. The resistance is normally caused by the mutations of gene in microbes. Many new drugs have been produced to encounter the problems, but the resistance develop by the bacteria is often quick while the production of new drugs is low with average of only four new drugs is produced every year. Bacterial resistance towards many antibiotics are increasing tremendously without any regards of class and mechanisms and it became very serious problem in the treatment of disease (Coates et al., 2002; Laxminarayan et al., 2013; Singh, 2014). Moreover, the usage of antibiotics may elicit undesirable side-effects such as diarrhea, vomiting and tooth staining according to Chung's 2006 study as cited in Palombo, (2011). Most of the commercially available mouthwashes consisting chlorhexidine and ethanol are normally leads to tooth staining and also has been linked to oral cancer respectively (Lachenmeier, 2008).

Plants extracts contains biologically active compounds and always been of great interest to researchers. It has been used thousands of years in the prevention and treatment of various diseases. *Ziziphus mauritiana* is a well-known traditional medicinal plant, found to have vast effect on various species of microorganisms. Based on the previous studies, it were found that *Z. mauritiana* has a very good antibacterial activities against many pathogens such as *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus sphericius*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsilla pneumoniae and Proteus vulgaris* (Abalaka, Daniyan, & Mann, 2010; Nagumanthri, Rahiman, Ahmad Tantry, Nissankararao, & Phani kumar, 2012; Siddiqui & Patil, 2015; Suman, 2012). A study done by Ghasham et al., (2017) in the recent years shows that *Z. mauritiana* contains various types of active compounds such as glycosides, coumarins, flavonoids, polyphenols, resins, saponins and tannins. Some of those active compounds are responsible as antimicrobial agents (Nagumanthri et al., 2012).

The aim of the present research was to investigate the effect of methanol extracts of *Z. mauritiana* on selected cariogenic bacteria. The finding from the current study is anticipated as a natural anti-bacterial alternative for oral health care.

## 2.0 METHODOLOGY

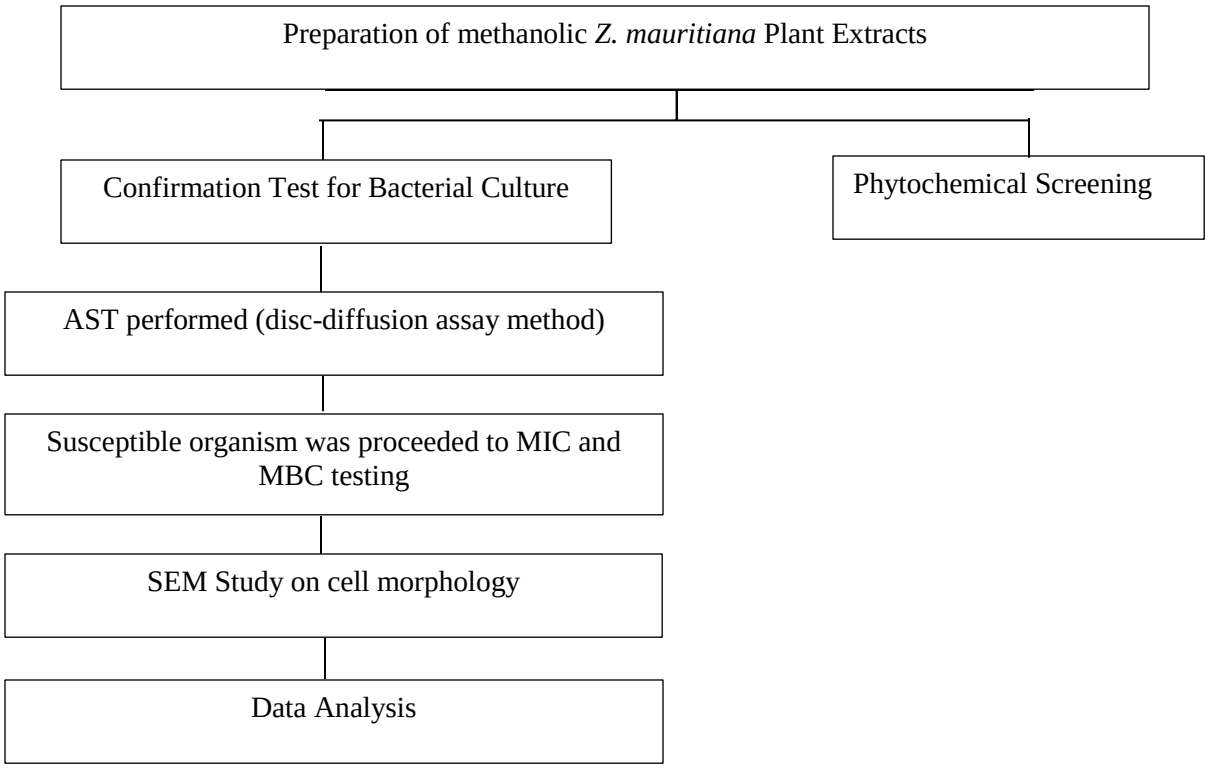


**Figure 1. Overview of methodology**

### 2.1 Preparation of Extracts

The dried powder of *Z. mauritiana* leaves was purchased from the local supplier (A). Methanol was used for the extraction of the plant. 100g of the dried powder of *Z. mauritiana* leaves was soak in 500 ml of methanol for 3 days with frequent soaking in a dark tighten container at room temperature (B). It was then filtered by using the Grade 1 Whatmann filter papers. The filtrate was then concentrated by using the rotary evaporator to remove all solvent (C). The crude extract was then collected and lyophilized using the freeze drier (D) and finally stored in the universal bottle before used (E).

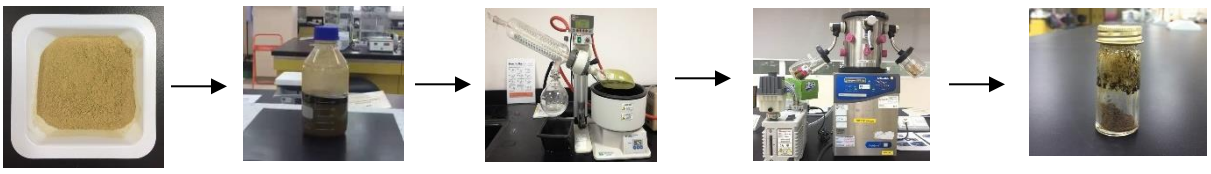


**Figure 2. Preparation of Extract**

2.2 Preparation of Stock Concentration

Powdered form of *Z. mauritiana* plant extract was weighed and suspended in 1 ml of 10% DMSO for tests performed in this study.

**2.3 Collection of Organisms**

*Streptococcus mutans* and *Streptococcus sobrinus* were obtained from the Centre of Preclinical Science Studies, Faculty of Dentistry, University Teknologi MARA, Sungai Buloh Campus. The organisms from the stock culture were inoculated on Brain Heart Infusion (BHI) agar and incubated at 37 ˚C for 18-24 hours. Then, the organism was subcultured into BHI broth and standardized to 0.08-0.13 using spectrophotometer (OD625nm) which is equivalent to approximately (1-2) $x10^6$ ~ (1-2) $x10^8$ CFU/mL for experimental purpose.

**2.4 Control Experiment**

Positive and negative control were prepared to compare the reaction and ensure the reliability of the result. 0.12% Chlorhexidine (mouthwash) were used as positive control and 10% DMSO were used as a negative control and let dried in the drying oven before use.

**2.5 Antibacterial Susceptibility Testing (AST)**

100 µl of the standardized inoculum suspension of the organisms were spread on the BHI agar by using sterile cotton swab. Blank discs impregnated with plant extracts were placed on the surface of the BHI agar aseptically and were gently pressed down to ensure contact. The plates were allowed to dry for 30 minutes prior to incubation at 35-37˚C for 20-24 hours.

**2.6 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)**

MIC and MBC values of plant extract were determined by micro-broth dilution assay method. The stock solution of plant extract was prepared by dissolving 200 mg of methanolic extract into 1 mL of 10 % DMSO and incubated at 37˚C for 18-24 hours. The microtiter plate was observed and solution from each well were streaked on the plates and incubated at 37˚C for 18-24 hours.

**2.7 Scanning Electron Microscopy (SEM) Study on Cell Morphology**

SEM was used to detect the cell morphological changes of the bacteria. Treated and untreated samples were analysed using SEM. Untreated sample was the bacterial culture without extracts. Treated sample was the bacterial sample incubated with the plant extracts (100 mg/ml). Both the treated and untreated sample were incubated at 37˚C for 18-24 hours. The sample was then sent to the Faculty of Pharmacy, UiTM Puncak Alam for SEM analysis.

**Note:** Organism that shows no zone of inhibition were not proceeded to MIC, MBC and SEM analaysis.

### 2.8 Preliminary Phytochemical Screening

The phytochemical activities were carried out according to Abalaka et al., (2010) and Najafi, (2013). The extracts were tested for alkaloid, phenols, tannins, flavonoids and saponin. The qualitative results were observed based on the reaction and expressed as presence or absence of phytochemicals.

### 2.9 Data Analysis

Data obtained from this study were analysed using Statistical Package of Social Sciences (SPSS) Version 21.0 by using descriptive statistics using mean and standard deviation (SD). All experiments were performed in triplicate.

## 3.0 RESULT ANALYSIS

### 3.1 Antibacterial Susceptibility Testing (AST)

Disc-diffusion assay of 500 mg/ml plant extracts were tested against *S. mutans* and *S. sobrinus* to study the antimicrobial properties of the plant and the results are shown in the Table 1 below.

**Table 1. AST results of *Z. mauritiana* plant extracts against *S. mutans* and *S. sobrinus***

| Organisms | Mean Zone of Inhibition (mm) | | |
|---|---|---|---|
| | Negative Control (10% DMSO) | Positive Control (0.12% Chlorhexidine) | Plant Extracts (500 mg/ml) |
| *S. mutans* | 6.00 ± 0.00 mm | 30.00±0.00 mm | 15.67±0.58 mm |
| *S. sobrinus* | 6.00 ± 0.00 mm | 26.43±0.40 mm | 6.00±0.00 mm |

6.00 mm: Diameter of Disc

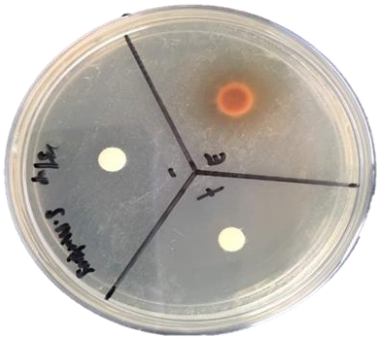

**Figure 3. AST of *S. mutans***

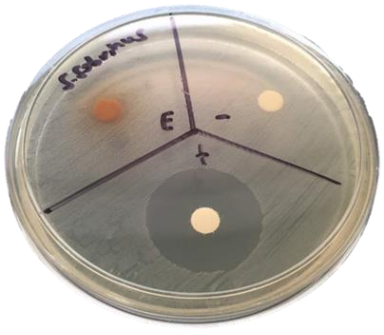

**Figure 4. AST of *S. sobrinus***

### 3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

*S. mutans* was then proceeded for MIC and MBC testing using broth-microdilution assay to obtain the bacteriostatic and bactericidal concentration of the plant extracts against the organism. *S. sobrinus* did not exhibit antibacterial properties (AST: 6.00±0.00 mm) and thus, was not proceeded for further analysis. Table 2 and Table 3 shows the MIC and MBC results for *S. mutans* respectively.

**Table 2. MIC of *S. mutans***

| | Concentration of Extracts (mg/mL) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Well | 200 | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0.78 | 0.39 | BC | IC | MIC |
| MP | C | C | T | T | T | T | T | T | T | T | C | T | 100 mg/ml |

Broth Control (BC), Inoculum Control (IC), Clear (C) = Indicates no growth of bacteria by naked eyes and Turbid (T) = Indicates bacterial growth

**Table 3. MBC of *S. mutans***

| | Concentration of Extracts (mg/mL) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Well | 200 | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0.78 | 0.39 | BC | IC | MBC |
| Plate | NG | G | G | G | G | G | G | G | G | G | NG | G | 200 mg/ml |

Broth Control (BC), Inoculum Control (IC), No Growth (NG) = Indicates no growth of bacteria by naked eyes and Growth (G) = Indicates bacterial growth

### 3.3 SEM analysis

The morphological changes of *S. mutans* was studied using Scanning Electron Microscope (SEM) and the results are shown in Figure 5(a) and Figure 5(b).

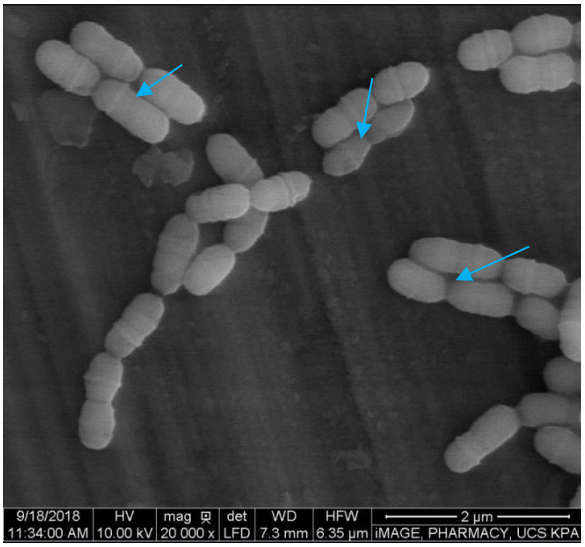


**Figure 5(a): Untreated *S. mutans***

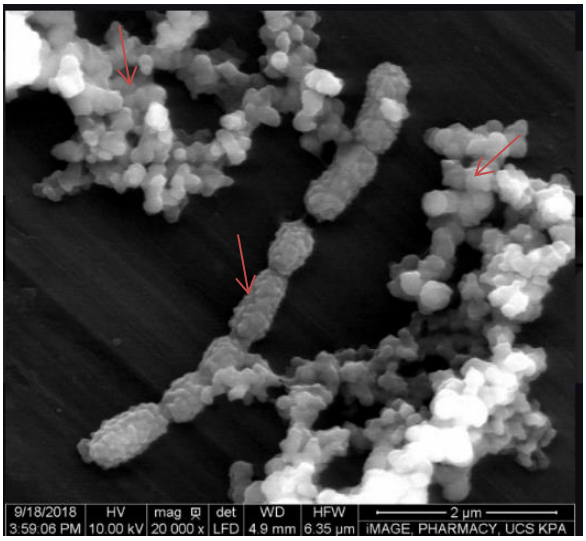


**Figure 5(b): Treated *S. mutans***

Untreated *S. mutans* were arranged in chain and covered by extracellular matrix (arrow in blue)[Figure 8(a)]. The blue arrow shows the extracellular polysaccharides matrix produce by *S. mutans*. Figure 8(b) shows the effect of the extracts on the cell morphology and extracellular polysaccharides formation surrounding the cell.

### 3.4 Preliminary Phytochemical Screening

The screening of phytochemical was conducted to study the secondary active metabolites that were present in the plant extracts. The results were presented in the Table 4.

**Table 4. Phytochemical Screening of the Plants**

| Phytochemicals | Reaction | Indication |
|---|---|---|
| Alkaloids | Formation of Brownish-red colour precipitation | Presence |
| Phenols | Formation of blue-black colour precipitation | Presence |
| Tannins | Formation of greenish-black colour precipitation | Presence |
| Flavonoids | Yellow colouration presence | Presence |
| Saponin | Persistence foamy layer for 15 minutes | Presence |

## 4.0 DISCUSSION

In the current study, the methanol extract of *Z. mauritiana* against *S. mutans* exhibited antibacterial activity with mean zone of inhibition of 15.67±0.58 mm for the AST analysis. The MIC and MBC values of *S. mutans* was 100mg/ml and 200mg/ml respectively. Scanning electron microscopic study demonstrated potential detrimental effect of the plant extracts on the morphology of *S. mutans*. Figure 8(a) shows untreated *S. mutans* and Figure 8(b) shows *S. mutans* treated with bacteriostatic concentration of plant extract at 100 mg/ml. The presence of extracellular polysaccharides matrix was observed surrounding the untreated *S. mutans* chains. However, extracellular polysaccharides matrix was absent for *S. mutans* treated with 100 mg/ml plant extracts. Besides the effect on the extracellular polysaccharides, it also affected the bacterial cell surface that lead to aggregation of the cells (Figure 8b). This finding suggests that the extracts had disrupted the cell wall of the selected bacteria. The loss of extracellular polysaccharides may have affected the attachment of the bacteria within the chain and lead to aggregation and clumping with other bacterial cells. Loss of polysaccharide formation will inhibit the adherence capacity of the *S. mutans* and eventually decrease the success rate of *S. mutans* to cause dental caries (Conrads et al., 2014). Other than aggregation, some of the treated bacterial cells surface were coated with the extracts (as shown in Figure 8b). The coating may have inhibited cell function and lead to cell death. Extracellular DNA (eDNA), proteins and polysaccharides are the major component of the bacterial biofilm, loss of formation of biofilm indicates that the plant extract may has disrupts the eDNA of *S. mutans* (Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002). The current study suggests that the methanol extract of *Z. mauritiana* has interfered with the formation of extracellular polysaccharides and disrupted the cell morphology that explains the antibacterial properties of the plant extracts (Yenugu, Hamil, French, & Hall, 2006).

On the contrary, there is no effect of methanol extract of *Z. mauritiana* against *S. sobrinus*. No inhibitory zone was observed for AST analysis. There are many factors that may have affected the antibacterial assay. This bacterium may require higher concentration of the plant extracts used. In addition, different active compounds extracted using different solvent also may play a major role in the activity of the plant extracts on bacteria cells. Effective compounds to penetrate and acts as antibacterial properties against *S. sobrinus* may not be presence in the plant extracts (Maharjan, Mainali, & Baral, 2011). Besides, different genetic constituent of different bacteria could be the other factor which caused the bacteria to resist the activity of the active compounds from the plant extracts. Studies reported that different bacteria has different genetic constituents and thus, gave different reactions when tested with the plant extracts (Ngoci, Ramadhan, Ngari, & Leonard, 2014). Moreover, the active compounds responsible for the antibacterial properties may have penetrate the cell wall and cell membrane structures of *S. sobrinus* at different rates compared to *S. mutans* (Nikaido, 2003). Thus, optimisation of the antibacterial activity of the plant extracts is required for *S. sobrinus*.

In the current study, the phytochemical screening for alkaloid, phenols, tannins, flavonoids and saponin of the methanol extract of *Z. mauritiana* leaves were performed. The phytochemical screening showed positive results to all five compounds tested and thus proves the presence of alkaloids, phenols, tannins, flavonoids and saponin in the plant extract. Other study conducted on phytochemical screening also showed the presence of active compounds demonstrated the antibacterial activity on the selected bacteria (Abalaka et al., 2010; Abdallah & Elsharkawy, 2016; Ghasham et al., 2017). Previous study has suggested that the

mechanism of action involved may have inhibited bacterial growth by various cellular process that disrupts membrane integrity and cause leakage of electrolyte from the cells (Walsh et al., 2003). Other than that the active compound present in the plant extracts may inhibit some of the bacterial enzyme or toxin activities and interferes the function of microbial DNA thus lead to cell death. (Omojate et al., 2014).

## 5.0 CONCLUSION AND FUTURE WORKS

In conclusion, the finding from the current study exhibit the potential of *Ziziphus mauritiana* plant extracts as antibacterial agent. The information gathered from this study would enable the scientific community to recommend the use of this naturally derived extract as an alternative to the commercially available oral health care products. Further study can be conducted using liquid chromatography-mass spectrophotometry (LCMS) and gas chromatography-mass spectrophotometry (GC-MS) analysis to determine detail analysis of the compound present in the plant extracts. Other than that, the transmission electron microscopy (TEM) study can be performed to study in depth the effect of the extracts on bacteria cell membrane and its contents.

## ACKNOWLEDGEMENT

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