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Original Article

The inhibitory effect of crude water extract of *Musa sapientum* inflorescence on *Streptococcus mutans*

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Abstract

Streptococcus mutans (*S. mutans*) has virulence factors involved in the pathogenesis of dental caries. The aim of this study was to investigate the inhibitory effects of the crude water extracts from *Musa sapientum* inflorescence (MSI water extract) on *S. mutans.* Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the broth microdilution method. In addition, the inhibitory effects on glycosyltransferase activity (GTFs) of *S. mutans*, biofilm formation, and acid production were examined. The MIC and MBC of MSI water extract were 62.5 and 125 mg/ml, respectively, tested with 0.05% v/v chlorhexidine as the positive control. At 2MIC, the viability of *S. mutans* was reduced by at least 2.3 log CFU/ml compared with the positive control. MSI water extract at MIC and sub-MIC showed more than 50 percent inhibitory effect on GTFs. MSI water extracts at 1/2MIC, 1/4MIC and 1/8MIC exhibited attenuation of the acid production in a dose-dependent manner. This results indicate that MSI water extract exhibits inhibitory effects on virulence factors of *S. mutans*.

Keywords: *Streptococcus mutans*, *Musa sapientum*, biofilm formation, acid production

1. Introduction

Dental caries results from an ecological imbalance in the physiological equilibrium between tooth minerals and oral microbial biofilms (Machiulskiene *et al.*, 2020). *Streptococcus mutans* (*S. mutans*) is an acidogenic bacterium considered to be the major causative agent of dental caries in humans (Krzyściak, Jurczak, Kościelniak, Bystrowska, & Skalniak, 2014). These bacteria are able to produce extracellular polymers and water-insoluble glucans from sucrose, which mediate their adherence on tooth surfaces and contribute to the glycolytic pathway and the formation of dental biofilms (Kutsch, 2014; Taso *et al.*, 2020). In the dental caries process, the tooth surface is covered with pellicles of protein, which is a soft coating generally invisible to the naked eye, harboring various microorganisms of oral flora including *S. mutans*. In the presence of fermentable carbohydrates, the acid produced by *S. mutans* starts eroding the superficial enamel or outermost tooth covering. However, due to the constant presence of salivary calcium and phosphate ions, the surface is remineralized continually and thus calcification occurs continuously. Two of the most important virulence factors of *S. mutans* are the ability to produce acid during the metabolism of dietary carbohydrates and the acid tolerance in dental plaque, which are essential for the development of dental caries. In addition, *S. mutans* ATCC 25175 is most commonly found in carious dentin, therefore, this species is representative of microbial dental caries (Vitorino *et al.*, 2004).

As a result, we can reduce the virulence factors of this species in dental biofilm by mechanical control; for

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example, by brushing and by chemical control using mouthwash or natural product mouthwash.

Natural products have gained more attention in the development of innovative therapeutic agents as they can be useful in alternative anticaries therapies. The *in-vitro* inhibitory activities of extracts or oils from various medicinal plants such as garlic, Alecrim-pimenta, grapefruit seeds, and many others against cariogenic and periodontopathic bacteria have been documented (Philip, Leishman, & Walsh, 2019). Therefore, there has been a similar interest in *Musa sapientum* inflorescence (MSI), which is a natural plant in Thailand.

MSI is a well-known medicinal plant belonging to the *Musaceae* family. It consists of a stalk, bracts and flowers (Lau *et al.*, 2020). MSI contains among its major components polyphenols and terpenoids (Lau *et al.*, 2020). Previous studies have shown that alkaline extract from flower of *Musa sapientum* can inhibit *Pseudomonas aeruginosa* (Sitthiya, Devkota, Sadiq, & Anal, 2018). In addition, water extract of MSI can potentially be a good source of antibacterial agents (Lau *et al.*, 2020).

However, there is little evidence to support efficacy of MSI in the prevention of dental caries (Ahmed *et al.*, 2021; Philip, Leishman, & Walsh, 2019). Therefore, the aim of this study was to investigate the effects of MSI water extract on bacterial viability, glycosyltransferase (GTFs) activity, adhesion, and acid production of *S. mutans* ATCC 25175.

2. Materials and Methods

2.1 Bacterial strain and culture medium

S. mutans ATCC 25175 are routinely maintained in our laboratory with weekly sub-cultures in Tryptic Soy (TS) Broth (BD, Sparks, MD, USA) containing 1% glucose (TSG broth) and the long-term storage is at -80ºC in TSG broth containing 10% v/v glycerol. To use these bacteria, *S. mutans* ATCC 25175 were resuscitated on mitis salivarius bacitracin agar at 37°C for 48 hrs. Typical colonies were re-inoculated in TSG broth at 37ºC for 16 hrs. Freshly grown microbial cultures were appropriately diluted to obtain the cell suspension at 10^6 CFU/ml.

2.2 Material: Plant material and preparation of extracts

MSI was purchased from a local market in Hat Yai, Songkhla, Thailand. The plants were identified by the morphology of the fruit and by the owner who sold the plants. The MSI was washed with distilled water, cut into small pieces, oven-dried at 55ºC for 2 days, and then ground into fine powder. Two hundred grams of the powdered material were boiled for 10 mins in 2 liter of distilled water and then stirred at 4ºC for 12 hrs on a rotatory platform. The resulting mixture was subsequently filtered through a cotton cloth and then vacuum-filtered through Whatman filter paper No.1 (W&R, England, UK). To ensure the sterilization, the extract was filtered through 0.45 µm Millipore paper (Millipore SAS, France) and then concentrated by freeze-drying. The extract was stored at −20ºC until use. A fresh 500 mg/ml stock solution was prepared prior to each experiment by reconstitution of the freeze-dried extract in distilled water. Therefore, this extract for the experiment is called crude water extract from MSI (MSI water extract).

2.3 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC and MBC of MSI water extract against *S. mutans* ATCC 25175 were determined using a micro-dilution technique (Zeng, Nikitkova, Abdelsalam, Li, & Xiao, 2019). A series of two-fold dilutions of MSI water extract ranging from 31.25 to 500 mg/ml were prepared in a volume of 100 µl per well in 96-well plates. TSG broth without any antibacterial agent was used as the negative control and TSG broth with 0.05% v/v chlorhexidine as the positive control. After addition of 100 µl of bacterial suspension ($1x10^6$ CFU/ml) into each well, the plate was vibrated for 1 min and then incubated at 37ºC for 24 hrs. The final concentrations of MSI water extract ranged from 15.625 to 250 mg/ml. MIC was defined as the lowest concentration at which no visible bacterial growth against TSG broth was observed (optimal density: OD < 0.05 at 550 nm). MBC was determined by subculturing the test dilutions on tryptic soy agar plates and incubation for a further 48 hrs. The lowest dilution that yielded no bacterial growth on solid medium was taken as the MBC. All assays were performed in triplicate and repeated 3 times.

2.4 The effects of MSI water extract on *S. mutans* ATCC 25175 growth

The MSI water extract was diluted to 2MIC, MIC, 1/2MIC, 1/4MIC, and 1/8MIC and combined with 10^6 CFU/ml, 600 µl of *S. mutans* ATCC 25175 in 2xTSG + 2% glucose for investigating the growth rate of *S. mutans*. The negative control was 600 µl deionized water. Next, evaluating the growth rate at 2, 4, 6, 8, 10, and 24 hrs was determined by counting the living *S. mutans* on the TS agar plate. All assays were performed in triplicate and repeated 3 times (Zeng *et al.*, 2019).

2.5 The effects of MSI water extract on the adhesion of S. mutans ATCC 25175

Briefly, overnight cultures of *S. mutans* ATCC 25175 were diluted in 2xTSG containing 1% W/V sucrose to approximately 1 x 10^7 CFU/ml. Two hundred microliters of two-fold serial dilutions of the MSI water extract were prepared in 48-well polystyrene plates, followed by the addition of 200 µl of diluted bacterial suspension. This resulted in final concentrations of the MSI water extract ranging from 7.81 to 31.25 mg/ml and final inoculums of 5 x 10^6 CFU in each well. TSG containing 0.5% w/v sucrose without any antibacterial agent was used as the negative control and TSG with 0.3% w/v sodium fluoride (NaF) was used as the positive control. The plate was incubated at 37°C for 24 hrs. After overnight culture, the media and planktonic cells were decanted from the plate. The remaining planktonic cells were removed by gently rinsing twice with sterile water. The biofilms were stained with 300 µl of 0.1% v/v crystal violet for 15 mins and rinsed thoroughly with sterile water twice. The bound dye was released from the cells with 300 µl

of 95% ethanol and the absorbance of the solution was measured at 560 nm using a microplate reader (Anthos Zenyth 200rt, Biochrom, UK). The relative amount of biofilm formed was calculated by comparing the OD560 of the test samples with those of the controls. The procedure was repeated three times (Zeng *et al.*, 2019).

2.6 The effects of MSI water extract on the GTFs of *S. mutans* ATCC 25175

Cultured *S. mutans* ATCC 25175 in cultures of 1-liter TSG broth at 37°C for 18 hrs were centrifuged at 6,000x *g* at 4°C for 30 mins to precipitate the cells. The supernatant was precipitated by adding 45% ammonium sulfate and placed at 4°C for 48 hrs, then centrifuged at 8,500x *g* in 4°C for 30 mins. The precipitate was dissolved in PBS, pH 7.4, dialyzed in PBS, pH 6.8, at 4°C for 48 hrs, and then lyophilized. The effects of MSI water extract on GTFs were tested at 1/2MIC, 1/4MIC, and 1/8MIC. The reaction mixture included 50 µl of 0.6 M acetate buffer (pH 5.5) + 50 µl crude enzyme + 200 µl of tested MSI water extract. In the negative control 200 µl of distilled water was used to replace the MSI water extract. Each concentration of MSI water extract without enzyme was used as blank. The reaction mixture was incubated at 37°C for 3 hrs, heated at 100°C for 5 mins then centrifuged at 13,000x *g* for 6 mins. The supernatant was removed and the pellet was washed twice with distilled water. The glucan was determined by adding 150 µl of 5% phenol and 750 µl of 99% sulfuric solution and heating at 110°C for 15 mins. The OD was measured at 490 nm. All assays were performed in triplicate and repeated 3 times (Zeng *et al.*,2019).

2.7 Effects on acid production

The effects of MSI water extract on the glycolytic pH-drop by *S. mutans* ATCC 25175 was determined as described elsewhere with a slight modification (Guo *et al.*, 2013). *S. mutans* ATCC 25175 were grown in TSG broth at 37°C for 18 hrs. The cells were harvested by centrifugation (6,500x *g* for 20 mins at 4°C). The cell pellet was re-suspended in 135 mM KCl (40 mg cells/ml) and incubated at 37°C under aerobic conditions for 3 hrs before use. The reaction mixture (5 ml) contained 1 ml of the bacterial cells (*S. mutans*), 3 ml of 135 mM KCl, 0.5 ml of 1% glucose, and 0.5 ml of MSI water extract (1/2MIC, 1/4MIC, or 1/8MIC). Sterile water was used as the negative control and 0.05% v/v chlorhexidine as the positive control. The initial pH of the mixtures was adjusted to the range 7.2-7.4 with 0.2 M KOH solution. The glycolytic pH-drop was monitored using a pH meter every 30 mins for 150 mins. The experiments were performed in triplicate.

2.8 Statistical analysis

All variables are presented as mean ± SD. To identify statistically significant differences the Kruskal-Wallis H-test was applied, followed by Dunnett's T3 for multiple comparison. The requirement of significance was set at $P < 0.05$.

3. Results and Discussion

3.1 The MIC and MBC of MSI water extract against S. *mutans* ATCC 25175

The antibacterial activity of MSI water extract against *S. mutans* ATCC 25175 was determined by broth microdilution. The MIC was 62.5 mg/ml and the MBC was 125 mg/ml compared with 0.05% v/v chlorhexidine as the positive control.

Different concentrations of MSI water extract namely 2MIC, MIC, 1/4MIC, and 1/8MIC were investigated for inhibition of *S. mutans* growth. The 2MIC, 125 mg/ml, had a significant inhibition on *S. mutans* growth from 4 hrs until 24 hrs. At 24 hrs after MSI water extract incubation, the amount of *S. mutans* had decreased by approximately 2.3 log CFU/ml compared to the control, as shown in Figure 1.

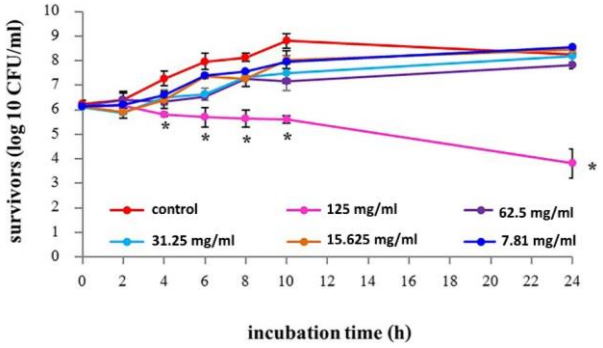

Figure 1. The effects of crude MSI water extract on *S. mutans* ATCC25175 growth in log CFU/ml (mean ± SD) * $p < 0.05$

S. mutans is a bacterium that plays a key role in causing caries and progressive carious lesions (Takashio & Okami, 1982). They adhere to the tooth surfaces and use GTFs enzymes to break down the food we eat. These bacteria then produce acid in the oral cavity through the digestion of carbohydrates. This results in the dissolution of minerals on the tooth surface leading to dental caries (Ooshima *et al.*, 2000). At present, the use of fluoride in the prevention of cavities is widely accepted and widely used because it is effective in preventing caries (Guha-Chowdhury, Iwami, Yamada, & Pearce, 1995). By helping to strengthen the structure of the teeth, it helps them withstand the acidic conditions in the oral cavity from the acidification by *S. mutans* and can also reduce the acidity of this type of bacteria (Van Loveren, 1990). However, it was also reported that at concentrations higher than 4 ppm it causes dental fluorosis (Jeng *et al.*, 1998). Therefore, medicinal plants may be a good alternative for caries prevention.

MSI extract contains alkaloids, glycosides, flavonoids, saponins, steroids, and tannins (Sahaa, Acharyaa, Shovon, & Royb, 2013; Zafar, Saleha, Hoque, & Sohel, 2011). Phenolic and phenol are oxidizing agents that inhibit enzyme activity by reacting with sulfhydryl groups of enzymes or by binding to non-specific microbial proteins (Cowan, 1999).

Methanol extract of *Musa paradisiaca cv. Mysore* inflorescences had the MIC values 16.5 and 31.0 mg/ml against *Staphylococcus aureus* and *Listeria monocytogenes*, respectively (Padam, Tin, Chye, & Ismail, 2012). Apart from banana inflorescences, the other parts of banana including leaves and fruit have shown antimicrobial activity. A prior study found that the extracts in ethyl acetate, methanol and hexane from banana leaves, *Musa sapientum*, *Musa acuminate* and *Musa paradisiaca* showed inhibitory activities. Ethyl acetate and methanol extracts from *Musa sapientum* banana leaves had MIC 125 µg/ml and MBC 250 µg/ml when tested against *Enterococcus faecalis* and *Staphylococcus aureus* (Karuppiah & Mustaffa, 2013). It was also found that methanol extract from *Musa acuminate* banana leaves had MIC 125 µg/ml and an MBC 250 µg/ml when tested with *Enterococcus faecalis* and *Staphylococcus aureus* by ethyl acetate extract from *Musa sapientum* banana leaves. Moreover, the ethanol extract from *Musa sapientum* banana fruit had approximately 50 mg/ml MIC while its MBC was approximately 60 mg/ml when tested with *Staphylo coccus aureus* and *Bacillus subtilis* (Fagbemi, Ugoji, Adenipekun, & Adelowotan, 2009). The juice extract from *Musa sapientum* banana fruit had a MIC of approximately 100 mg/ml when tested on *S Staphylococcus aureus* and *Bacillus subtilis* (Fagbemi *et al.*, 2009). In addition, a study conducted by Sahu *et al.* (2015) tested the MIC and MBC of *Musa sapientum* banana leaf juice extract against several pathogenic and resistant Gram-positive bacteria: *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylo coccus saprophyticus*, *S. mutans*, and *Staphylococcus. pyogenes*. They were found to have MIC 3.12 mg/ml and MBC 6.25 mg/ml when tested against *Enterococcu faecalis*, and when tested against other strains *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *S. mutans*, and *Staphylococcus pyogenes* were found to have the same MIC at 1.56 mg/ml and MBC value of 25.50 mg/ml with no MBC, but with 3.25 mg/ml and 6.25 mg/ml, respectively (Sahu, Dubey, Rath, Panda, & Padhy, 2015).

Sahu *et al.* (2015) found that extracts from raw banana pulp (*M. sapientum L. subsp. Sylvestris*) inhibited both Gram-positive and Gram-negative bacteria that cause disease, diarrhea and dysentery (Sahu *et al.*, 2015). The possible mechanisms may involve inhibitory alkaloids. Then this ingredient is inserted between the DNA strands, inhibiting bacterial DNA production. At the same time, it may involve tannin which is capable of binding to enzymes by hydrogen bonding or nonspecific binding. In addition, tannin is membrane toxic and can bind to substrates of infection as well (Zafar, Saleha, Hoque, & Sohel, 2011). The activity of flavonoids extracted from *Nidus Vespae*, a plant found in China, is often used as an herbal remedy for toothache. It has antimicrobial activity, and was found to inhibit the growth of *S. mutans* by means of antagonist flavonoids, possibly permanently binding to extracellular proteins. As a result, the protein function is disrupted (Guan *et al.*, 2012).

Therefore, the extracts from different parts of banana had MIC values in the range 6.83 µg/ml - 280 mg/ml and MBC in the range 62.50 µg/ml - 280 mg/ml. The differences in our results from the others may be due to the source and variety of the plant, different bacterial strains tested, and different solvents used to extract.

3.2 The effects of MSI water extract on the adhesion, GTFs and acid production of *S. mutans* ATCC 25175

The MSI water extracts were studied at various concentrations namely MIC, 1/2MIC, 1/4MIC, and 1/8MIC, and 0.1% w/v NaF was used as the positive control. Inhibiting the adhesion of *S. mutans* on 48 well plates was tested by the broth microdilution method at 24 hrs, and turbidity measurements at 560 nm are shown in Figure 2. The concentrations 62.5 (MIC), 31.25 (1/2MIC) and 15.625 (1/4MIC) mg/ml significantly inhibited *S. mutans* adhesion in contrast to the negative control group ($p < 0.05$) by 52.94%, 84.16% and 44.45%, respectively, while 0.1% w / v NaF, which was the positive control, could inhibit the adhesion of bacteria by 98.14%.

The efficacy of MSI water extracts at the concentrations MIC, 1/2MIC, 1/4MIC, and 1/8MIC was investigated in relation to the enzyme GTFs of *S. mutans* by determining the sugar content. The Phenol-sulfuric method was used to measure the absorbance at 490 nm as shown in Figure 3. (1/8MIC) 7.81 mg / ml was statistically significant in inhibiting GTFs of *S. mutans* relative to the control group ($p < 0.05$). It was able to inhibit the enzyme GTFs of germs at 94.72%, 63.23 and 59.80%, respectively. The extract at 62.5 mg/ml (MIC) could not be measured for absorbance because the substance had a black color; however the lower concentrations did not interfere. The interference with absorbance measurements is a limitation of this method.

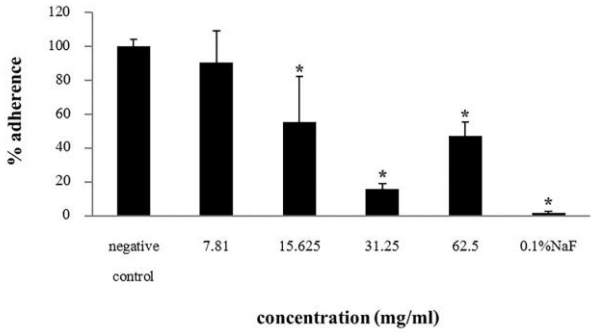

Figure 2. The effects of crude MSI water extract on *S.mutans* ATCC 25175 adhesion (mean ± SD, $p < 0.05$)

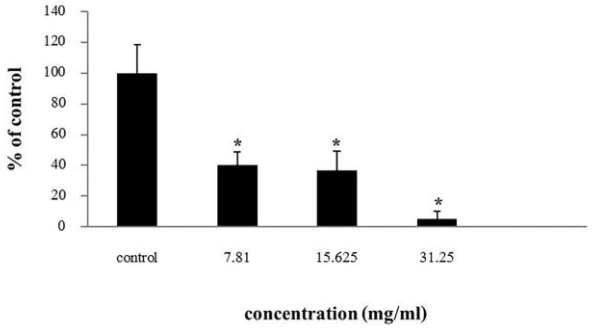

Figure 3. The effects of MSI water extract on GTFs of *S.mutans* ATCC 25175 (mean ± SD, $p < 0.05$)

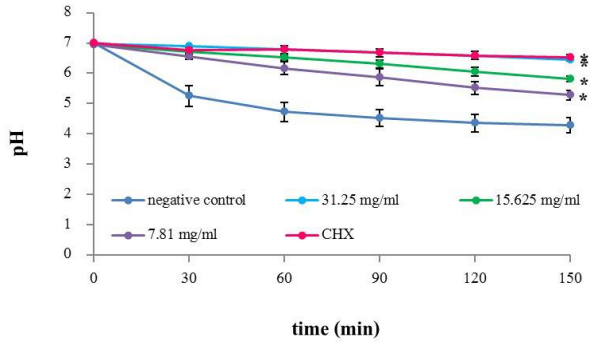

Figure 4. Effects of crude MSI water extract on acidification by *S. mutans* ATCC 25175 (mean ± SD). * indicates a statistically significant difference ($p < 0.05$) compared to the negative control group

The efficacy of crude MSI water extract at the concentrations 1/2MIC, 1/4MIC and 1/8MIC was studied using 0.05% v/v chlorhexidine as positive control. The ability of the extract to inhibit the acidification by the bacteria depends on the concentration and duration. The pH was significantly different from the negative control from 30 mins onwards with the crude extracts from bananas at all tested concentrations. At 60 mins and at 150 mins the pH was significantly different from the negative control group. It was found that all concentrations of crude extracts from MSI reduced the acidity.

The ability to form insoluble glucans through the degradation of nutrients we consume allows the bacteria to adhere to the teeth and create growing dental plaque (Ooshima *et al.*, 2000). Plant-derived polyphenols were reported to inhibit acid-producing bacteria, in particular inhibiting the growth of *S. mutans* and inhibiting GTFs (Ferrazzano *et al.*, 2011). In studies conducted by Jagtap and Karkera (2000), water and alcohol extracts from plants of the *Juglandaceae regia* group were shown to inhibit the adhesion and insoluble glucan formation of *S. mutans* by tannins. Changes to the hydrophobic properties (hydrophobicity) on the cell surface at an infection can inhibit enzymes produced by microorganisms by binding to the active sites of the insoluble enzyme and changing the structure of that enzyme. All of these results corroborate the prevention of adsorption of GTFs on the cell surface of *S. mutans* (Jagtap & Karkera, 2000).

S. mutans is the leading bacterium that can cause dental caries. It has virulence factors such as the release of a wide variety of acids including lactic acid and formic acid (Bowen, 1998). Acetic acid is obtained from the digestion of carbohydrate foods when the oral cavity is in a state with a small amount of sugar. Bacteria break down sugars into acetic acid and formic acid causing a slight decrease in oral pH but no damage to the structure of the teeth. However, when there is a lot of sugar in the mouth, bacteria produce lactic acids. Lactic acid is produced from glycolytic pathways by lactate dehydrogenase and fructose-1.6 a-diphosphate (FDP), and is an important factor. It reduces the pH within the plaque, which leads to the dissolution of minerals on the tooth surface (Dashper & Reynolds, 1992; Leme, Koo, Bellato, Bedi, & Cury, 2006). Therefore, reducing the amount of oral bacteria or reducing these virulence factors is important in prevention of caries. For this study, the test was controlled in a state of high sugar content. The test results show that MSI water extract at a concentration lower than the MIC (sub-MIC) can inhibit acid production by this bacterial species. MSI water extract at a concentration of 31.25 mg/ml (1/2MIC) can reduce the production of bacterial acidity. The production of bacterial acidity included lactic acid, formic acid and acetic acid from the glycolytic pathway of fermentation in high glucose consumption.

The possible mechanisms of biochemical constituents within MSI water extracts are briefly discusses. MSI water extract had as potentially active components polyphenols and terpenoids (Lau *et al.*, 2020). The polyphenols and terpenoids are well-known as anti-microbial agents (Gyawali & Ibrahim, 2014; Lau *et al.*, 2020). The polyphenols and terpenoids inhibit lactate dehydrogenase of fructose-1,6-diphosphate and amylase in saliva (Dashper & Reynolds, 1992; Lau *et al.*, 2020; Leme, Koo, Bellato, Bedi, & Cury, 2006). These mechanisms by the active components may contribute to suppressing the virulence factors of *S. mutans.* Therefore, the MSI water extract preventing plaque formation and reducing acid production by *S. mutans* can be understood by these mechanisms.

4. Conclusions

The results of the present study indicate that MSI water extract is a promising natural anticariogenic agent that exhibits inhibition of growth, bacterial adhesion, GTFs activity and acid production by *S mutans.* However, this study was done *in vitro*, which differs from the actual oral conditions especially in oral bacterial diversity, saliva flow, and tooth surface appearance. In future studies, the active ingredients of the plant and the mechanisms of action should be investigated.

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