Antimicrobial Property of *Zingiber officinalis* Extract on *Streptococcus mutans*

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Abstract

Zingiber officinalis (Z. officinalis) from Apiaceae family is Ayurveda medicine in Asia. Z. officinalis is traditionally used as herbal, food, and various therapeutic purposes. This study aimed to determine the antimicrobial activity of Z. officinalis crude extract against Streptococcus mutans (S. mutans), in-vitro.

Using the broth dilution method, the antibacterial activity of Z. officinalis crude extract was evaluated in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Biofilm development was used to investigate the inhibitory effect of Z. officinalis extract on bacterial adhesion. The amount of water-insoluble glucan was used to determine the inhibitory effect of crude extract on glucosyltransferase (GTFs). In addition, the acidity of S. mutans pathogenesis was measured using sub-MIC level of Z. officinalis extract.

The MIC and MBC of Z. officinalis crude extract against S. mutans were 31.25 and 62.50 mg/ml, respectively. Biofilm formation was inhibited at MIC and 1/2 MIC. Extracts of Z. officinalis at MIC level inhibited GTFs by more than 50%. In addition, extracts of Z. officinalis at 1/2 MIC, 1/4 MIC, and 1/8MIC showed a dose-dependent reduction in acid production.

Z. officinalis extract can inhibit the virulent factor of S. mutans, which causes dental caries in humans, in vitro.

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Introduction

Zingiber officinalis (*Z. officinalis*) , also known as ginger, is a spice used in both culinary and medicine around the world. The Ginger contains phenolic compounds (gingerol and shogaol) , sesquiterpene hydrocarbons, and oleoresins, which are its main active ingredient.[1] Consequently, the ginger us responsible for its medicinal properties, including antiarthritis, antiinflammatory, antidiabetic, antibacterial attributes[2,3]. Given the increased occurrence of antibiotic resistance as a result of usage and harmful effects, medicinal plants are currently being researched for treatment against bacterial diseases including dental caries.[4]

Dental caries, often known as tooth decay, is caused by pathogenic bacteria in plaque on the tooth surface, which converts sugar into lactic acid by glycolytic pathway, damaging the tooth surface over time[5,6]

Significant bacterial group causing dental caries is *Streptococcus mutans* (*S. mutans*) . In addition to the acidic qualities of *S. mutans*, this bacteria might survive in acidic environments with a pH of up to 3.[7] *S.mutans* can adhere to the tooth surface by generating extracellular polymer binders. [8] Glucosyltransferase (GTF) from *S. mutans* plays role in convert glucose into extracellular matrix including insoluble glucans. The aforementioned steps allow *S. mutans* to colonize and adhere the tooth surface, leading to dental biofilm accumulation[9] This process dissolves enamel and dentin and it has a continuous impact on the occurrence of caries.

Brushing your teeth and using chemical control are two strategies that may be utilized in the fight against the development of dental caries. Because natural products have the potential to be effective in alternative anti-caries treatments, there has been an increased focus on their potential application in the development of novel therapeutic agents[10] It has been

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demonstrated that extracts and oils derived from a wide variety of medicinal herbs, including garlic, grapefruit seeds, and *Lippia sidoides*, amongst many others, suppress the growth of cariogenic and periodontopathic bacteria when tested *in vitro* study.[11,12] In addition, the extract of *Z. officinalis* displays significant antibacterial action against *Streptococcus* cariogenic germs.[13]

Owing to the potential benefit of *Z. officinalis*, the objective of this study was to investigate the effect of extracts *from Z. officinalis* on bacterial viability, adhesion, glycosyltransferase (GTFs) activity, and acid production of *S. mutans*.

Materials and methods

Bacterial strain and culture medium

S. mutans ATCC 25175 was maintained with weekly sub-cultured in Tryptic soy (TS) Broth (BD, Sparks, MD, USA) containing 1% glucose (TSG broth) and long-term storage at -80ºC in TSG broth containing 10% v/v glycerol. *S. mutans* ATCC 25175 was resuscitated on mitis salivarius bacitracin agar for 48 hrs at 37ºC.Typical colonies were reinoculated in TSG broth. Freshly developed microbial cultures were diluted to yield 10^6 CFU/ml of cell suspension.[12]

Preparation of extracts

Z. officinalis obtained from a local market in Hat Yai, Songkhla, Thailand, were washed with distilled water and squeezed in juicer extractor machine ZO Extract was passed through filter cloth, centrifuged and then filtered steriled with 0. 45 μ m Millipore filter paper (Millipore SAS, France). The crude extract was freeze-dried and kept at -20°C until used. Before used, the lyophilized powder was resuspended in deionized water at 500 mg/mL.

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

Using a microdilution technique, the MIC and MBC of ZO extract against *S. mutans* ATCC 25175 were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[14] The stock solution was serially diluted ranging from 3.9 to 62.5 mg/ml. Chlorhexidine 0.05% v/v was used as a positive control. One hundred microliters of the diluted extracts and 100 μL of approximately 1×10^5 CFU/mL bacterial suspension were added into 96 well-plate and cultured at 37°C. After 24 hrs, the lowest concentration which displayed no visible growth was recorded as MIC. The MBC was the lowest extract concentration killing 99.9% of the bacteria inoculum after 24 hrs incubation at 37 °C.[12,15,16] The experiments were done in triplicate.

The effect of ZO extract on the adhesion of *S. mutans* ATCC 25175

Briefly, approximately 1 x 10^6 CFU/ ml overnight cultures of *S. mutans* ATCC 25175 were diluted in 2xTSG containing 1% w/ v sucrose. One hundred microliters of two-fold serial dilutions of the ZO extract were prepared in 96-well plates, followed by the addition of 100 μl of diluted bacterial suspension. This resulted in final concentrations of the ZO extract ranging from 3.9 to 31.25 mg/ml and final inoculums of 5 x 10^5 CFU in each well. TSG containing 1% w/v sucrose without any antibacterial agent was used as the negative control and TSG with 0.2% w/v NaF was used as the positive control. After 24 hrs cultured, the media and planktonic cells were decanted from the plate and then gently rinsed twice with sterile water. The biofilms were stained with 100 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 200 μl of 95% ethanol. 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 OD 560 of the test samples with the controls. The experiment was repeated three times.[15,16,17]

The effect of ZO extract on the GTFs of *S. mutans* ATCC 25175

GTFs were prepared from 1-liter of cultured *S. mutans* ATCC 25175. The supernatant was collected by centrifugation and the GTFs was precipitated by adding 45% ammonium sulfate. After placed at 4ºC for 48 hrs, the precipitate was centrifuged at 8,500x g in 4ºC for 30 mins. The precipitate was subjected to dissolve in PBS, pH 7.4, dialyzed in PBS, pH 6.8, at 4ºC for 48 hrs, and then lyophilized. The effects of ZO extract on GTFs were examined at 1/ 2MIC and 1/ 4MIC. The reaction mixture contained 50 μl of 0.6 M acetate buffer (pH 5.5) + 50 μl crude enzyme + 200 μl of tested ZO extract. Distilled water was used to replace the extract as a negative control. Each concentration of ZO extract without enzyme was used as blank. The reaction was set at 37°C for 3 hrs, then

heated at 100°C for 5 mins and 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 phenol-sulfuric method [18] by adding 150 µl of 5% phenol + 750 µl of 99% sulfuric solution and then heating at 110°C for 15 mins. The OD was read at 490 nm. The assays were done in triplicate and repeated 3 times. [15, 16, 17]

The effect of ZO extract on the acid production of *S. mutans* ATCC 25175

The effects of ZO extract on the acid production by *S. mutans* ATCC 25175 was examined according to Nattapon R. *et al.* (2022) . [15]After 18 hrs cultured, cells were collected by centrifugation at 6,500x *g* for 20 mins at 4°C and washed twice with PBS, pH 7.4. The cell pellet was resuspended at 40 mg cells/ml in 135 mM KCl and incubated at 37°C for 3 hrs before used. The reactions were set up by adding 1 ml of *S. mutans*, 3 ml of 135 mM KCl, 0.5 ml of 1% glucose, and 0.5 ml of ZO extract at 1/2MIC or 1/4MIC.

Sterile water was used as a negative control and chlorhexidine at a final concentrations of 0.05% v/v as the positive control. The initial pH of each reaction was adjusted between 7.2-7.4 with 0.2 M KOH. The glycolytic pH-drop was monitored using a pH meter at 30, 60, 90, 120 and 150 min. The experiments were set up 3 times.[15,19]

Statistical analysis

All variables were represented with means ± SD. To calculate the significant difference of parameters, the Kruskal-Wallis H test was applied, followed by Dunnett T3 for multiple comparison. The level of significance was set at $p < 0.05$.

Results

The MIC and MBC of ZO extract against S. *mutans* ATCC 25175

MIC and MBC were used to investigate antimicrobial activities of ZO extract. The MIC of ZO extract against *S. mutans* was 31.25 mg/ml and the MBC value was 62.50 mg/ml. These results showed that ZO extract could inhibit *S. mutans* proliferation and survival.

The effect of ZO extract on biofilm formation of *S. mutans* ATCC 25175

The effect of ZO extract on biofilm formation was done at MIC and sub MIC level. We found that at MIC and 1/2 MIC of ZO extract significantly inhibited *S. mutans* adhesion by 81.54% and 66.55%, respectively, comparing to negative control, whereas 0.1% w/v NaF could inhibit bacterial adhesion by 94.61% , $p < 0.05$ (Fig. 1).

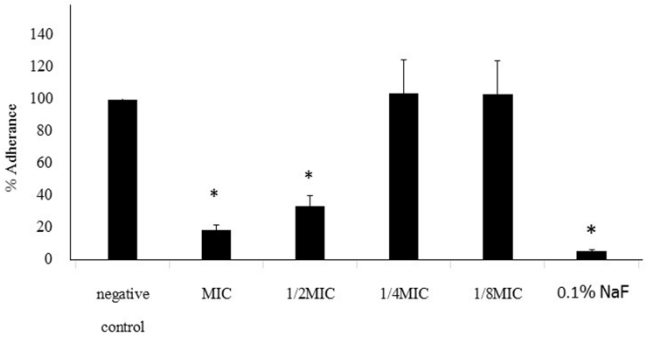

Figure 1. The effect of crude extract from ZO on S. mutans ATCC 25175 adhesion (mean ± SD)* showed a statistically significant difference ($p < 0.05$) compared to the negative control group.

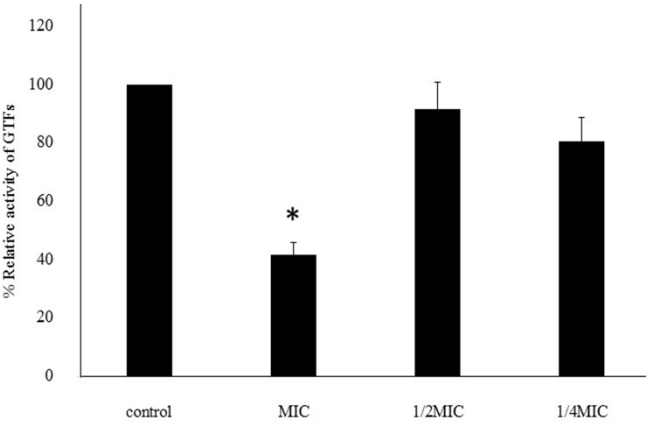

Figure 2. The effect of crude extract from ZO on GTFs of *S. mutans* ATCC 25175 (mean ± SD)* showed a statistically significant difference ($p < 0.05$) compared to the negative control group.

The effect of ZO extract on GTFs of *S. mutans* ATCC 25175

Phenol sulfuric acid method was used to determine the amount of extracellular polysaccharides which was a necessary factor for *S. mutans* adherence. We found that MIC of ZO extract significantly inhibited GTFs activity by 58.74% comparing to the control, $p<0.05$ (Fig. 2).

The effect of ZO extract on acid production of *S. mutans* ATCC 25175

As shown in Figure 3, ZO extract could significantly inhibit the glycolytic acid production by *S. mutans* at 1/2 MIC, 1/4 MIC, and 1/8MIC comparing to the negative control, $p<0.05$. The onset pH 7.00 was decreased to 4.67 after 30 min of incubation in the control group. Whereas, in 1/2 MIC, 1/4 MIC and 1/8 MIC of ZO treatment groups, the pH was raised to 6.7, 6.47, and 6.06, respectively. At 150 mins, the pH value was significantly higher than the negative control group ($p < 0.05$).

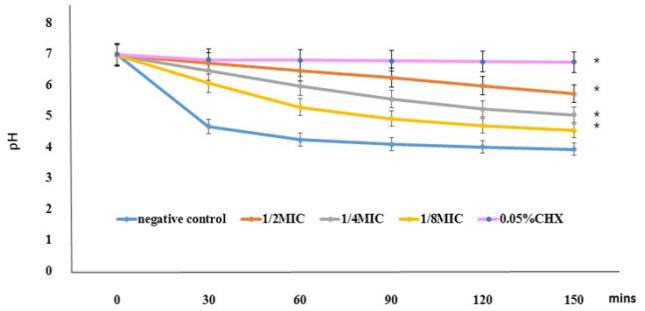

Figure 3. The effect of crude extract from ZO on acidification of *S. mutans* ATCC 25175 (mean ± SD). * showed a statistically significant difference ($p < 0.05$) compared to the negative control group.

Discussion

This study offers *in vitro* experimental evidences that ZO extract showed antimicrobial activity against *S. mutans*. Based on previous evidences, the major component of ZO extract is phenolic compound which is D-nerolidol, α Curcumen, Tran- 10 – Shogaol, Paradol[20,21]

These components may be responsible for the antimicrobial activity against *S. mutans*. Our study is consistent with previous reports showed that *Z. Officinale* water extract had showed 0.02 mg/ml as MIC and 0.04 mg/ml as MBC to *S. mutans* .[13] Both *Z. Officinale* crude extract and methanolic fraction extract had showed 256 µg/ml. to *S. mutans*.[21] Furthermore, the nerolidol ,which is a component in ZO extract, found in other plant such as *Musa sapientum* flower. Our study found that a crude water extract of *Musa sapientum* flower inhibits the virulence factors of *S. mutans.*[15]

The probable strategies of this herbal against *S.mutans* could be caused by weakening the membranous tissue of the microorganism's cell wall.[22] However, the MIC and MBC values in the prior study were different. The variation could be attributed to the plant component used, the type of microbial strains used, the solvents used, and the chemical constituents.

The interaction of sucrose and microbial adherence is one of the most important step in biofilm formation. GTFs is play a role in the conversion of sucrose to insoluble glucan, which promotes the breakdown of nutrients permits the bacteria to adhere to surfaces and proliferate dental biofilm formation.[23]

The reduction in insoluble glucan causes a decrease in biofilm formation as it will interfere with integrity and stability, reducing the availability of binding site for *S. mutans*. It is evident form the results that MIC and sub-MIC of ZO extract reduced biofilm formation and GTFs activity. Similar to other study had reported that crude extract and methanolic fraction of *Z. officinale* had a significant inhibition of biofilm formation via *S. mutans* .[21] The possible mechanism had stated that *Z. officinale* extract ,which contains nerolidol and flavonoid, can alter exopolysaccharide matrix resulting in ineffective *S. mutans* adherence[21]

The ability to suppress the biofilm formation by several natural extracts can occur by lowering the action potential of the biofilm mass so that the biofilm does not develop and is degraded from the membrane surface. In addition, it also inhibits the production of extracellular substances such as insoluble glucan, so that it can disrupt the ion channel communication between bacterial species involved in the formation of biofilms.[24] According to the hypothesized mechanisms, the phenolic molecule may alter the hydrophobic characteristics of the infected cell surface, which then binds to the active site of the insoluble enzyme leading to an inhibition of caries progressing.[25,26]

Furthermore, *S. mutans* is an aciduric bacterial which can cause a glycolytic pH drop. The decreased pH in oral cavity is less than 5.5 which cause enamel caries progression. Our study found that sub-MIC of ZO extract contribute to an inhibition of a glycolytic pH value, marked by a tremendous reduction in the initial and final rate of the pH. The previous study had reported that phenolic compound might cause a reduction of lactate dehydrogenase and fructose-1.6 a-diphosphate (FDP) which is a key enzyme for *S. mutans* glycolytic pathway.[27]

However, our research was performed *in*

vitro, which means that the results may not be directly applicable to real oral environment, such as the diversity of oral bacteria, the flow of saliva, or the appearance of tooth surfaces. Also, the exactly mechanisms of caries prevention by herbal should be further investigated.

Conclusions

ZO extract can inhibit the virulent factor of *S. mutans*, which causes dental caries in humans, *in vitro* study. However, the active components in this extract and its pharmacological property should be further study

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Declaration of interest

The authors have no conflicts of interest relevant to this article.

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