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ORIGINAL ARTICLE

Ginger Essential Oil Inhibits Biofilm Formation of *Streptococcus mutans* on Stainless Steel Placeholder

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ABSTRAK

Streptococcus mutans (S. mutans) membentuk biofilm pada peralatan dan bahan pergigian dalam mulut iaitu permukaan gigi. Pembentukan biofilm ini boleh menyebabkan pereputan gigi. Dalam kajian ini, pembentukan biofilm pada penyelenggara ruang keluli tahan karat, yang sering digunakan oleh kanak-kanak dan perencatan oleh minyak pati halia telah dikaji. Asai kepekatan perencat minimum (MIC) dijalankan untuk mengkaji aktiviti antibakterial minyak pati halia mengikut kepada teknik larutan broth. Pembentukan biofilm pada penyelenggara ruang keluli tahan karat dinilai dengan menggunakan spektrofotometer dan bilangan bakteria dalam biofilm telah dihitung. Biofilm pada penyelenggara ruang keluli tahan karat juga dikaji dengan menggunakan mikroskop elektron pengimbas (SEM). Pada penyelenggara ruang keluli tahan karat yang tidak mempunyai minyak pati halia, S. mutans menghasilkan lebih banyak biofilm. Bilangan bakteria dan gambar SEM juga menyokong dapatan ini. Minyak pati halia (Zingiber officinale) menghalang pembentukan biofilm dan pelekatan S. mutans pada pemegang tempat keluli tahan karat.

Kata kunci: aktiviti antibiofilem, karies, halia, minyak pati Zingiber officinale (Roscoe) Streptococcus mutans

ABSTRACT

Streptococcus mutans (*S. mutans)* forms biofilm on dental apparatus and materials in the mouth as far as the tooth surface is concerned. This form of biofilm around the apparatus can cause decay in the teeth. In our study, the formation of biofilm

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on the stainless-steel space maintainer, which is frequently used in children, and its inhibition by ginger essential oil were examined. Minimum inhibitory concentration (MIC) assays for the antibacterial activities of the ginger essential oil was performed according to the microdilution broth method. The formation of the biofilm on the stainless steel space maintainer was evaluated spectrophotometrically and the number of bacteria in the biofilm was counted. Biofilm on stainless steel space maintainer was also examined with scanning electron microscopy (SEM). On the stainless steel space maintainer without ginger essential oil, *S. mutans* produced significant amounts of biofilm. Bacterial counts and SEM images also supported this finding. Ginger (*Zingiber officinale)* essential oil significantly inhibited the formation of biofilm and adhesion of *S. mutans* on the stainless-steel placeholder.

Keywords: antibiofilm activity, caries, ginger, *Streptococcus mutans*, *Zingiber officinale* (*Roscoe*) essential oil

INTRODUCTION

The human mouth is a very complex and dynamic structure. More than 700 bacterial species have been identified in the oral cavity. Adhesion and colonisation of oral bacteria on tooth surfaces result in the formation of a biofilm containing many bacterial species called dental plaque (Aas et al. 2005; Avila et al. 2009; Dewhirst et al. 2010). Biofilm formation causes common oral infectious diseases such as dental caries, gingivitis, periodontitis and peri-implantitis. Plaque occurs not only on natural teeth, but also on dental materials and implant components. Biofilm formation is a defence method of microorganisms against various stress conditions. Microorganisms in the biofilm are resistant to disinfectants and antibiotics. They are also not affected by nutrient deprivation, pH changes and oxygen radicals (Donlan & Costerton 2002).

Streptococcus mutans (*S. mutans)* is one of the early colonising bacteria in biofilm formation (Bowen & Koo 2011). *S. mutans* forms biofilms on other apparatus and materials in the mouth, as far as the tooth surface is concerned. This form of biofilm can cause decay in the teeth around the apparatus. *S. mutans* are one of the most important factors of dental plaque and tooth decay. As microorganisms metabolise the dietary carbohydrates, acids are formed. Acids cause the mineralisation of teeth by lowering the pH (Bowen & Koo 2011; Wade 2013). *S. mutans* produces glucose-transferase, a water-insoluble glucan from sucrose in food, a synthesising bacterial extracellular polysaccharide (EPS) (Bowen & Koo 2011). Some chemicals have been investigated to prevent biofilm formation. However, it has been reported that most of these substances are not effective enough to prevent biofilm formation. Chemicals such as fluoride and chlorhexidine can inhibit biofilm formation, but certain

concentrations of these substances are known to be cytotoxic (Baehni & Takeuchi 2003).

Ginger (*Zingiber officinale* Roscoe), is one of the most frequently used medicinal plant. Many bioactive compounds have been isolated from different parts of the plant (Mahboubi 2019). Ginger essential oil contains high levels of α-Zingiberene, β-sesquiphellandrene, (E,E)-α-farnesene, geranial and ar-curcumene (Mahboubi 2019). The biological activities of ginger have been studied extensively. Many studies have been revealed pharmacological activities, such as, antioxidant, a antimicrobial, antinociceptive, antimutagenic, anti-inflammatory, anticonvulsant, analgesic, antiulcer, gastric antisecretory, antidiabetic, nephroprotective, antitumor, anticancer, antispasmodic, antithrombotic, and hepatoprotective, hypocholesterolemic, antiallergic, antiserotonergic, anticholinergic, antioxidant, larvicidal and immunomodulatory (Ali et al. 2008; Syafitri et al. 2018; Aleem et al. 2020; Kausar et al. 2021). There are studies about the antibacterial effect of ginger against a number of microorganisms including *S. mutans* (Sa'ada et al. 2015; Mahboubi 2019; Aleem et al. 2020). However, there are only few reports for evaluating its antibiofilm potential (Ali et al. 2008; Bellik 2014). There are few studies in the literature on the adhesion of *S. mutans* on stainless steel area protectors. The purpose of this study is to assess the effect of *S. mutans* on biofilm formation, on the stainless-steel area protectors that are used in children with ginger essential oil.

MATERIALS AND METHODS

Microorganism

S. mutans was provided from the Microbiology Department of the Faculty of Science, Eskisehir Technical University. The *S. mutans* culture was maintained in 20% glycerol at -86°C. Prior to use, the culture was activated. The culture was used in studies after its purity and viability were confirmed.

Plant Material and Preparation of Essential Oil

Ginger rhizom was purchased from a local market in Eskişehir, TURKEY. Ginger essential oil was obtained by extraction with Clevenger type apparatus for 3 hours. Analysis of ginger essential oil was done by gas chromatography mass spectrometry (Agilent HP-7890B GC, German, 5977B Mass Selective Detector System, USA).

Saliva Sample

Saliva sample was obtained from a patient (16 years old girl) who did not have any systemic disease and had not received any antibiotic treatment in the last 2 months before the procedure. Five milliliter of fresh saliva was collected 2 hours after meal. The saliva sample was centrifuged at 10,000 rpm for 30 minutes at 4°C. It was then kept in a 56°C water bath for 30 minutes. The supernatant was used after being

filtered through a 0.22 filter with a syringe.

Stainless Steel Space Maintainer

The steel wire specimens were cut in 1 cm length 0.7 mm in diameter, steel wire rope (Universal, Silberlot Isprlgen, German), and the end of the wire was trimmed with large-sized carbon separator (0.4 mm) and rolled with metal rubber. The steel wire and band saws were soldered with steel wires cut into 1 cm long and 0.7 mm in diameter so that the inclinations of the bands were suitable for the second molar teeth used in children (interface and buccal face connection). Then the surplus was taken with large size carbon separator and the edges were rounded using metal tires. Wire samples were polished in a polishing motor using a polishing paste (Oropol, Detax, Ettlingen, German). They were bathed in hot steam to get rid of the remaining debris. Before use, metal wire and tape samples were autoclaved at 121°C for 20 minutes.

Determination of Bacteriostatic (MIC) and Bactericidal (MBC) Concentration

Minimum inhibitory concentration (MIC) assays for the antibacterial activities of the *Zingiber officinale* essential oil was performed according to the microdilution broth method of the National Committee for Clinical Laboratory Standards (CLSI) 2013. The lowest ginger essential oil concentration (MIC) was determined, which inhibits bacterial growth. The dilution without bacterial growth on Mitis Salivarus (MS) Agar was determined as minimum bactericidal concentration (MBC).

In vitro Biofilm Assay

Biofilm production of *S. mutans* was determined by Congo red agar (CRA) (Christensen et al. 1985) and microtitration plate method (Freeman et al. 1989; Stepanovic et al. 2000). Slime production by the isolates was determined by CRA method as described by Freeman et al. (1989). *S. mutans* was inoculated on the medium prepared by adding 0.08% Congo red to brain heart infusion (BHI) agar containing 1% lactose. Plates were incubated at 35°C for 18-24 hours in conditions containing 10% CO_2. Black colonies were considered positive, and pink-red colonies that did not change color were evaluated as negative (Christensen et al. 1985).

To determine biofilm by microtitration; BHI broths containing 2% of glucose, lactose, fructose, galactose, raffinose, maltose and sucrose were prepared separately. From the 18-24 hours fresh cultures of the *S. mutans*, the wells of the ELISA plate containing different sugars were inoculated at a ratio of 1:40. Only the medium was added to the negative control wells. Plates were incubated at 35°C in an environment containing 10% CO_2 for 48 hours. Then, the medium in the plate was emptied and the plate was washed three times with sterile phosphate buffered saline (PBS). 200 µl of 95% methanol was transferred onto the plate and left for

15 minutes. Then the methanol in the plate was drained and the plate was dried for 15 minutes. After drying the wells, 200 µl of 2% crystal violet dye solution was added and left for 5 minutes. Then the dye in the plate was drained and washed three times with PBS. Then, 160 µl of 33% (v/v) glacial acetic acid was added to the wells and read at 570 nm wavelength in a spectrophotometer (Shimadzu, UV-2101PC). The experiments were repeated 3 times (Freeman et al. 1989; Stepanovic et al. 2000).

Antibiofilm Activity of Ginger

The biofilm of *S. mutans* was formed on saliva-coated stainless steel space maintainer in batch cultures. The prepared stainless steel space maintainers were put into saliva with 2 ml of phosphate-buffered sterile water (PBS). The tubes were kept at 36°C for 20 minutes (Cardoso et al. 2012). Stainless steel space maintainers were removed from saliva and washed with sterile PBS and placed in 24-well polystyrene plate, one for each well. The 24-hour of *S. mutans* culture (10^{-6} cfu/ml) developed in BHI broth containing 2% sucrose was transferred onto each stainless-steel space maintainer. A total of 21.25 µl/ml (MIC) of ginger essential oil was added to a group of stainless-steel space maintainers. Then, BHI broth containing 2% sucrose was transferred on it.

In another group of stainless-steel space maintainers, BHI broth containing only 2% sucrose was added. All plates were incubated under anaerobic conditions for 48 hours at 35°C.

At the end of the incubation period, the biofilm formed on stainless steel space maintainers were evaluated spectrophotometrically. For this, the stainless-steel space maintainer was carefully removed from the medium and carefully washed 3 times with PBS. The stainless steel space maintainer was placed in a plate and dried, then stained with 1% crystalline violet for 15 minutes and washed again with PBS. 200 µl of glacial acetic acid was added to the stainless steel space maintainer to dissolve the crystal vial bond. The optical density was read at 570 nm in a microplate reader by transferring to a multi-well plate (Pitts et al. 2003).

For bacterial count; as described above, sterile stainless steel space maintainer on which biofilm formed were carefully removed and washed with PBS. Then, each stainless steel space maintainer was transferred to a 15 mL tube containing sterile glass beads of 1 mm diameter and 1 mL of PBS was added. The tubes were shaken by vortexing at 2500 rpm for 1.5 minutes. Cells were separated from the biofilm matrix. Dilutions were then prepared, and bacteria was counted by the drip plate method. All studies were carried out in triplicates.

Scanning Electron Microscopy (SEM)

The biofilm on the stainless steel space maintainer was also examined by SEM. The method by Okajima et al. (2006) was applied with minor modifications. The stainless steel space maintainers were washed with 0.1 M cacodylate

buffer. Then it was fixed with 2.5% glutaraldehyde at room temperature for 1-1.5 hours and washed again with cacodylate buffer. Then, 1 hour post-fixation was done with 1% osmium tetroxide (OsO_4). It was washed again 2-3 times with Cacodylate buffer. Dehydration was performed with alcohol series (30%, 50%, 70%, 90%, and 100%). It was dried in a Critical Point Dryer and plated with gold and analysed via SEM.

Statistical Analysis

T-test was used to compare bacterial counts and biofilm values obtained from the stainless steel space maintainer. P values of less than 0.05 were considered statistically significant.

RESULTS

The ginger that we used in the test contains 1.05% essential oil. The main constituent was zingibere (21.76%) and sesquiterpene. (Sesquiterpenes; β-sesquiphellandrene, 9.29%; bisbolene 5.32 % and farnesene, 5.20%). β-phellandrene content as a monoterpene was 1.51%.

The MIC value of ginger essential oil was determined as 21.25 µl/ml. It was observed that ginger essential oil had no bactericidal effect on *S. mutans*. Biofilm formation of *S. mutans* was observed on all sugar, except maltose (Table 1). Weak biofilm was formed in raffinose. High biofilm formation was observed in other sugars. By CRA method, *S. mutans* were slime producers developing black colonies on CRA plate (Figure 1).

S. mutans produced high amounts of biofilm on the saliva-coated stainless steel space maintainer. *Z. officinale* essential oil (21.25 µl/ml) inhibited the formation *of S. mutans* biofilm on the surface of saliva-coated stainless steel space maintainer (Figure 2 & 3). At MIC value, ginger essential oil inhibited the biofilm on the stainless steel space maintainer by 89.16%. Ginger essential oil was found to be effective against biofilm formation of *S. mutans* ($P<0.05$).

Bacterial counts were significantly reduced in samples added with ginger (Figure 3). A statistically significant difference was also found in terms of *S. mutans* counts ($P<0.05$). The effect of the *Z. officinale* essential oil on structural integrity of biofilm was also observed by scanning electron microscopy (Figure 4). The SEM data of *S mutans* biofilm formations were similar to the bacterial count data and values determined by the spectrophotometer.

The formation of biofilm on stainless steel space maintainers was examined in a scanning electron microscope (SEM). Strong biofilm formation was observed in controls, but no strong

Table 1: Biofilm formation in different sugar

Bacteria	Glucose	Fructose	Galactose	Lactose	Maltose	Raffinose	Sucrose
S. mutans	+++	+++	+++	+++	-	+	+++

non-adherent (-), weakly (+), moderately (++), or strongly (+++) adherent

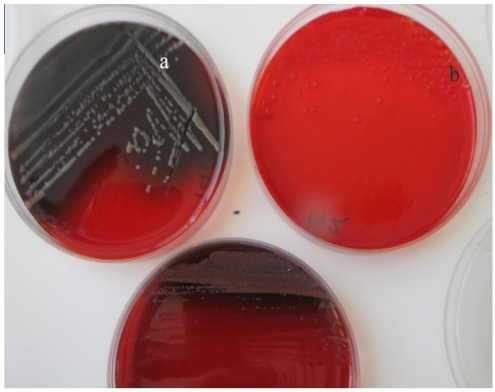

Figure 1: Growth of *S. mutans* strains on CRA plates

biofilm formation was observed in stainless steel space maintainers treated with ginger essential oil (Figure 4).

DISCUSSION

Plants have played an important role in the protection of human health since ancient times. In recent years, there has been an increased interest in plants and their bioactive compounds. Ginger is a plant that has been used for a long time. Because it is a natural compound, it can be used easily in children until a certain concentration.

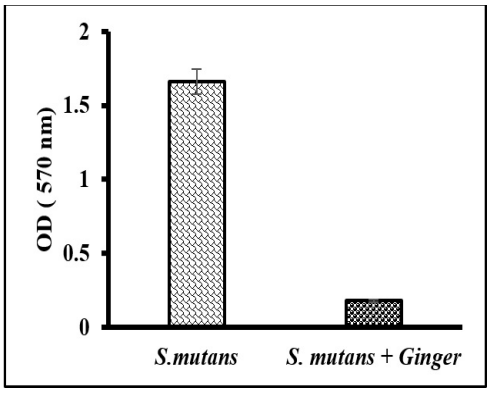

Figure 2: Biofilm formation of *S. mutans* on saliva-coated stainless steel space maintainer

The antimicrobial activity of ginger extracts and essential oil had been demonstrated previously (Stoyanova et al. 2006; Malu et al. 2009; Das et al. 2019). In this study, we found that ginger essential oil has antibacterial activity on *S. mutans*. The MIC of the essential oil of *Z. officinale* against *S. mutans* was found to be 21.25 µg/mL. Ginger methanol extract was reported to inhibit *S. mutans* at a concentration of 256 µg/mL (Hasan et al. 2015). Azizi et al. (2015) reported that ginger extract had antibacterial activity against *S. mutans* and *S. sanguinis* cariogenic microorganisms. They reported the minimum inhibitory concentrations (MICs) of ginger extract as 0.02 mg/mL for *S. mutans* and 0.3 mg/mL for *S. sanguinis*. The minimum bactericidal concentration (MBC) was reported as 0.04 mg/ml for *S. mutans* and 0.6 mg/ml for *S. sanguinis*. It had been reported that 2,500 mg/mL (MIC) ginger significantly inhibited the growth of *S. mutans* (Lau 2017). Similarly, crude ginger and methanol extracts were reported to be effective on *S. mutans* (Rios et al.1988). Researchers reported that in kinetic killing assay, extracts

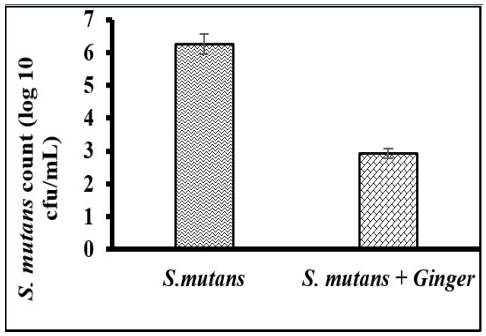

Figure 3: Bacteria counts on saliva-coated stainless steel space maintainer with biofilm formation

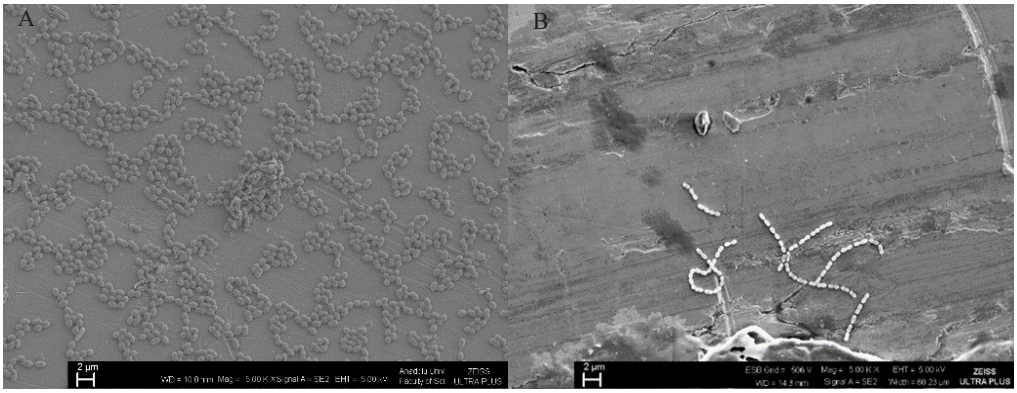

Figure 4: Electron microscopy (SEM) of biofilms of *S. mutans* without (A) and with (B) *Z. officinale* essential oil (21,25 µl/ml) exposure

caused a rapid decrease in bacterial counts. In this way they reported that oral cavity dental decay could be avoided. The antibacterial activity of ginger extracts could be attributed to the chemical properties of ginger. The main constituents of ginger are sesquiterpenoids with zingiberene (α-zingiberene and β-sesquifellandrene) and other sesquiterpenoids presenting in smaller amounts such as bisabolene and α-farnecene. Other components include sesquiterpenoids (β-sesquiphellandrene, bisabolene and farnesene), and trace monoterpenoid (β-sesquiphellandrene, cineol and citral) (O'Hara et al. 1998). It has been found that bisabolene and isocaryophyllene in the content of ginger essential oil exhibit antibacterial activity (Sharma et al. 2016). In our study, 9.29% sesquiphellandrene and 5.32% bisabolene were detected in the ginger essential oil. Therefore, we postulated that antibacterial activity may be provided by these compounds.

S. mutans produced strong biofilms on sugars other than maltose and raffinose. Dwivedi and Singh (2016) examined the biofilm formation of 30 *S. mutans* isolates. They reported that 18 isolates were strong, 9 isolates were medium-biofilm producers, and 3 isolates were not biofilm producers (Dwivedi & Singh 2016).

On the stainless steel space maintainer, *S. mutans* produced significant amounts of biofilm. Biofilm production of *S. mutans* was significantly suppressed in the presence of 21.25 µl/ml ginger essential oil (Figure 2). The essential oil damages the cell wall and membranes of microorganisms, changing the morphology and coagulating the cytoplasmic material. In addition, they have good anti-biofilm and anti-quorum sensing (QS) effects (Kumar & Anad 1998). *S. mutans* possess a variety of virulence factors to form biofilm such as QS systems, surface adhesins, extracellular polysaccharides, and glucan-binding proteins. The synthesis of water-insoluble glucans plays an important role in biofilm formation (Krzyściak et al. 2014). Ginger essential oil can affect biofilm formation by reducing glucan synthesis. *S. mutans*

secretes glucosyltransferases (GTFs). The GTFs encoded by the gtfB, gtfC and gtfD genes synthesise glucans using sucrose (Bowen & Koo 2011). Prevention of GTFase will reduce biofilm formation. The reduction of *S. mutans* biofilm formation with the application of ginger essential oil may be due to decreased expression of Gtf-encoding genes (gtfB, gtfC, and gtfD) (Xu et al. 2011). In the sucrose-bound adhesive, adhesions are provided by the synthesis of glucans. The GTFs enzyme plays an important role here. Prevention of GTFase will reduce biofilm formation

The most important step in biofilm formation is the surface adhesion of *S. mutans*. Terpenes in ginger essential oil may have an effect on the prevention of adhesion of *S. mutans* on stainless steel space protector. Similar findings were also reported by Das et al. (2019). Moreover, cellular adhesion may be due to hydrophobic interactions, or may be sucrose-bound adhesion (Matsumoto et al. 1999). Bacteria can not bind when the hydrophobic interaction between cell and surface decreases. Contrary to our findings, Lau (2017) reported that 2.5 mg/ml ginger had no effect on the biofilm of *S. mutans*. In another study, researchers found that ginger methanol extract inhibited the adhesion of *S. mutans*. Researchers have determined that ginger extract inhibits glycosyltransferase activity (Matsumoto et al. 1999).

In addition, ginger essential oil may have inhibited exopolysaccharide (EPS) synthesis of *S. mutans*. This may lead to a decrease in biofilm formation. Zhang et al. (2021) showed that *Ligustrum robustum* (Roxb.), Blume extract inhibited both bacterial growth and EPS synthesis, resulting in less accumulation of *S. mutans* cells and EPS.

CONCLUSION

The MIC (21.25 µl/ml) value of ginger essential oil inhibited biofilm formation on the stainless steel space maintainer by 89.16%. Ginger essential oil can be used to prevent dental caries by preventing biofilm formation of cariogenic *S. mutans*. It can be used especially in children with a stainless steel space maintainer in their mouth. However, it is important to confirm this finding with in vivo studies.

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