In vitro Antibacterial Activity of *Zingiber officinale* and *Orthosiphon stamineus* on *Enterococcus faecalis*

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Abstract

This study evaluates the antibacterial effects of *Zingiber officinale* essential oil and *Orthosiphon stamineus* water extract against *Enterococcus faecalis*. The herbs were prepared in various concentrations to determine their minimum inhibitory concentrations (MIC) and growth inhibitory effect. Anti-adhesion activities of the herbs were determined by co-incubation with *E. faecalis* cultures for 6 and 24 h. Biofilm disruption activities were determined by adding the studied herbs into preformed *E. faecalis* biofilm. The effects on the morphology of *E. faecalis* grown as biofilm were studied using scanning electron microscopy (SEM). The MICs of ginger oil and *O. stamineus* extract were 0.31 and 25 mg/mL, respectively. Between the tested herbs, ginger exhibited greater inhibitory effects on the growth of *E. faecalis* grown in suspension mode. Both herbs generally showed anti-adhesion activities in inverse concentration-dependent manner. No significant biofilm disruption activities by both herbs were observed. SEM analyses showed *E. faecalis* cell surface changes in the treated biofilm. The studied herbs may have compromised the integrity of the bacterial cell membrane. These findings suggest that the studied herbs may have better antibacterial activities against *E. faecalis* in suspension mode compared to biofilm mode, with ginger oil showed greater antibacterial activity compared to *O. stamineus* extract.

Keywords: *Zingiber officinale*, *Orthosiphon stamineus*, *Enterococcus faecalis*, antibacterial

1. Introduction

Dental caries and periodontal disease are two of the most prevalent oral health problems worldwide (Kassebaum et al., 2017). The main cause of these conditions is bacterial infection from the dental plaque. Amongst the common pathogenic species involved in both conditions are *Enterococcus faecalis*, a facultative anaerobic bacteria and notoriously known to be resistant to antibacterial and clinical therapy (Stuart et al., 2006). Recent findings in drug discovery research using herbal extracts have given new hopes for supplemental or even alternative approach to managing dental infections more effectively and safely.

Zingiber officinale (ginger) from the *Zingiberaceae* family, is an aromatic rhizomatous plant grown throughout Asia and tropical regions (Sasidharan & Menon, 2010; Imtiyaz et al., 2013). It has been used as medicine for diarrhoea (Imtiyaz et al., 2013), and nausea caused by motion and morning sickness, as well as chemotherapy (Lete & Allué, 2016). The active components of ginger such as gingerol, volatile oil, shaogol and diarylheptanoids have been reported to exhibit anti-inflammatory, antioxidant, antilipid, antidiabetic, analgesic, antipyretic and anticancer activities (Kim et al., 2008; Sasidharan & Menon, 2010; Shim et al., 2011, Imtiyaz et al., 2013, Ghasemzadeh et al., 2016). Ginger has also been reported to exhibit strong antibacterial effects against several pathogens such as *Escherichia coli*, *Proteus* spp., *Staphylococcus* spp. (Karuppiah & Rajaram, 2012), *Streptococcus spp* (Sebiomo et al., 2011; Azizi et al., 2015), *Pseudomonas aeruginosa* (Kim & Park, 2013) and *Candida albicans* (Takahashi et al., 2011). Recently, there have been reports on the use of ginger in dentistry (Ficker et al., 2003; Atai et al., 2009; Patel et al., 2011).

Orthosiphon stamineus, also known as "misai kucing" (cat's whiskers) in Malay, is a medicinal herb belonging to the family *Lamiaceae*. *O. stamineus* is widely used traditionally for the treatment of various disorders, such as those affecting the urinary tract, diabetes, gout, rheumatism and jaundice (Perry, 1980). The beneficial *O. stamineus* chemical compounds are polymethaxylated flavonoids such as sinensetin, eupatorine and caffeic acids derivatives, which include rosmarinic acid, cichoric acid and caffeic acid (Tezuka et al., 2000; Olah et al., 2003; Mohamed et al., 2012). It has been reported that 50% methanolic extract of *O. stamineus* showed protective

effects against alcohol-induced gastropathy, liver damage, pyrexia (Yam et al., 2007, 2009a, 2009b), anti-inflammatory and analgesic effects (Yam et al., 2008). Studies on the antimicrobial activity of *O. stamineus* have been reported against *Staphylococcus aureus*, *Streptococcus agalactiae* and *Vibrio parahemolyticus* (Ho et al., 2010; Mohammed et al., 2012).

Although these studies have shown the beneficial effects of both plants, little is reported on the antibacterial properties of these herbs in the field of dentistry, especially on bacteria implicated in periodontal and endodontic diseases like the notoriously resistant *Enterococcus faecalis*. This study will help to appreciate the antibacterial properties of *Z. officinale* and *O. stamineus* toward *E. faecalis* in suspension culture as well as in biofilm environment. Therefore, the aim of this research was to investigate the antibacterial effects of *Z. officinale* and *O. stamineus* on *E. faecalis*. The findings on the structural changes of *E. faecalis* after exposure to these two herbs, using scanning electron microscopy (SEM) was also reported.

2. Materials and Methods

2.1 Preparation of Ginger and O. stamineus

Ginger essential oil was obtained from the Drug Discovery Research Laboratory of Universiti Kebangsaan Malaysia (courtesy of Dr. Shahida Mohd Said). Briefly, ground ginger was boiled for 8 h in the boiling flask with a tight-fitting adapter attached to the aspirator vacuum. The volatile components of the samples were evaporated with steam and cooled as oil at the end of the procedure. The oil was then collected, and remaining water was removed using anhydrous sodium sulphate. *O. stamineus* powder was obtained from Faculty of Bioprocess, Universiti Teknologi Malaysia, Skudai, Johor (courtesy of Associate Professor Dr. Adibah Abdul Majid).

2.2 Bacterial Strain and Growth Conditions

E. faecalis ATCC 29212 was used in this study. The bacteria was grown on brain heart infusion (BHI) agar (Oxoid, UK) and passaged weekly. Cultures on BHI agar or in BHI broth (Becton Dickinson, USA) were incubated for 24 h at 37 $^{\circ}$C in the presence of 5% CO_2 with 95% relative humidity (Shel Lab).

2.3 Broth Microdilution Assays

A range of two-fold dilutions of ginger oil in dimethyl sulfoxide (DMSO) (Acros Organics) and *O. stamineus* extract in purified water were prepared in sterile 96-well plates and inoculated with a standardized suspension of bacteria and incubated for 24 h. There were eight concentrations of ginger oil (5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04 mg/mL) and *O. stamineus* extract (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg/mL) used in the viability assays described below, using broth microdilution technique.

The positive controls that were used to compare with the tested herbs were dilution series of amoxicillin or ampicillin (Sigma, USA), and 2.5% sodium hypochlorite (NaClO). Amoxicillin or ampicillin was used because they are common antibiotics clinically used for patients with any periodontal, endodontic or perio-endo infections and NaClO is a common irrigant solution used in clinics to clean the root canal system due to its antibacterial properties.

After 24 h of exposure to studied herbs, the optical density (OD) of the growth turbidity was measured with a plate reader at 590 nm wavelength (Varioskan® Flash Microplate reader, Thermo Scientific, USA). The minimum concentration of the tested herbs that inhibits the growth of the *E. faecalis* after 24 h of incubation was recorded as the MIC. The growth inhibition of *E. faecalis* grown in suspension mode by the tested herbs was expressed as the ratio relative to positive control, NaClO.

2.4 Anti-Adhesion Assay and Biofilm Disruption Test

For the anti-adhesion assay, both *E. faecalis* cultures (1×10^5 CFU/mL) and the studied herbs were co-incubated into sterile 96-well plates in the anti-adhesion test for 6 and 24 h as described previously. In biofilm disruption test, *E. faecalis* biofilm was pre-grown in 96-well plates for 48 h. The prepared herbs were added to the preformed biofilm and further incubated for 24 h. Untreated *E. faecalis* acted as the negative control, while ampicillin (5 mg/mL) and 2.5% NaClO-treated biofilm acted as the positive controls for both tests. The biofilm formation in both tests was quantified using crystal violet staining. The absorbance of growth turbidity was measured with a plate reader at 590 nm wavelength.

2.5 Quantification of Biofilm by Crystal Violet Staining

Biofilm formation by *E. faecalis* was quantified according to Yamada et al. (2005) with modifications. The media containing unattached cells was gently pipetted out. Two hundred microliters of 0.1% crystal violet solution (Sigma, USA) was added and incubated for 15 min at room temperature. The crystal violet solutions

were gently pipetted out and the wells were washed three times with purified water. Two hundred microliters of 80% ethanol/20% acetone was later added. The solution was incubated for 10 min at room temperature. One-hundred microliters of the ethanol/acetone solution was transferred from each well to the new 96-well plates. The absorbance was measured with a plate reader at 590 nm.

2.6 Scanning Electron Microscopy (SEM)

Ginger oil, *O. stamineus* extract, ampicillin and NaClO were added into bacteria culture and dispensed into 24-well plates where cut glass slides were placed. After incubation for 48 h, glass slides bearing the attached *E. faecalis* cells were taken out and fixed with 2% glutaraldehyde using vapour fixation technique for 24 h at room temperature. After the fixation, the cells were rinsed several times with 0.1 M phosphate buffered saline (pH 7) for a minimum of 10 min. The cells were then dehydrated through a series of ethanol rinses (50%, 70%, 90% and 100%) for 10 min each. Finally, the cells were treated with hexamethyldisilazane for 10 min, followed by air drying. The glass slides were attached to aluminium mounting stubs and sputter coated with gold. The morphology of the attached bacteria and control was examined and viewed under field emission scanning electron microscope (FESEM) (Zeiss Supra 55VP) using various magnifications.

2.7 Statistical Analysis

IBM-SPSS version 22 was used for the statistical analyses. The mean and the standard deviation of the absorbance readings were obtained, and independent-sample T-test was carried out to compare the mean values between the studied herbs and the controls. The level of significance was set at $p < 0.05$.

3. Results

3.1 MICs of The Tested Herbs, and Inhibition of E. faecalis Growth

The MICs of ginger oil and *O. stamineus* extract against *E. faecalis* were 0.31 and 25 mg/mL, respectively. The inhibition of *E. faecalis* grown in suspension mode by ginger oil was directly concentration-dependent, with 0.313 mg/mL of ginger oil showed significantly greater inhibition compared to NaClO (Figure 1a). In general, *O. stamineus* extract showed greater inhibition for all the tested concentrations, compared to NaClO, although none of the tested concentrations exhibited significant growth inhibition compared to by NaClO (Figure 1b).
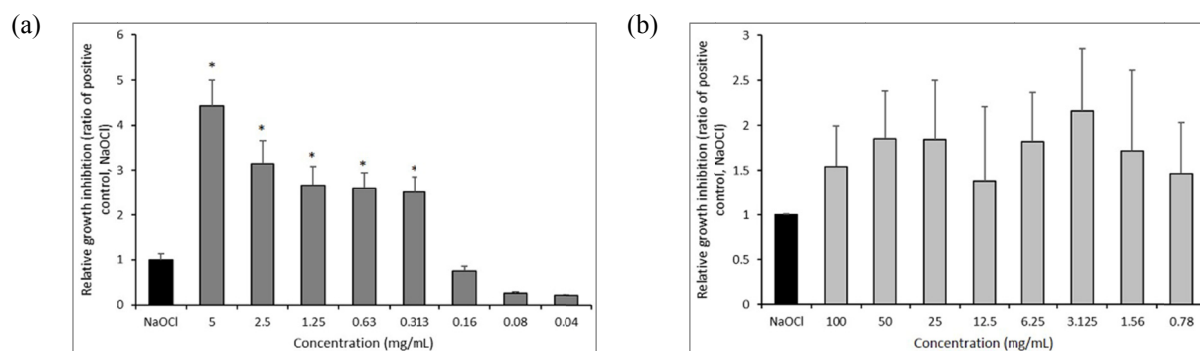

Figure 1. Growth inhibition of *E. faecalis* grown in suspension mode, following 24 h exposure with ginger oil (a) and *O. stamineus* extract (b)

Note. The growth inhibition for all the tested concentration was expressed as relative to inhibition by the positive control, 2.5% NaClO. Error bars represent the mean ± SD of two independent experiments performed in triplicates. * Significant differences ($p < 0.05$) between the ginger oil with 2.5% NaClO.

3.2 Anti-Adhesion and Biofilm Disruption Activities

Against *E. faecalis*, ginger oil exhibited inverse concentration-dependent anti-adhesion activity for both 6 and 24 h of exposure time (Figure 2a). Significant anti-adhesion activity was observed at lower ginger oil concentrations (0.04-0.08 mg/mL) compared to treatment with NaClO. Comparatively, longer exposure time (24 h) showed greater anti-adhesion activity for almost all the tested ginger oil concentrations. For *O. stamineus* extract (Figure 2b), a 6 h exposure produced inverse concentration-dependent anti-adhesion activity for concentrations 0.78-12.5 mg/mL. Conversely, the higher concentrations (25-100 mg/mL) showed a direct concentration-dependent

anti-adhesion activity on *E. faecalis*. Similar to ginger oil, the 24 h exposure resulted in inverse-concentration dependent anti-adhesion activity against *E. faecalis* for all *O. stamineus* extract concentrations.

For biofilm disruption activities, ginger oil showed inverse concentration-dependent effects (Figure 2c), similar to its post-6 h exposure anti-adhesion activity. *O. stamineus* extract showed inverse concentration-dependent activity at concentrations 0.78-6.25 mg/mL, before the biofilm disruption activity increases at concentrations 12.5-50 mg/mL (Figure 2d).
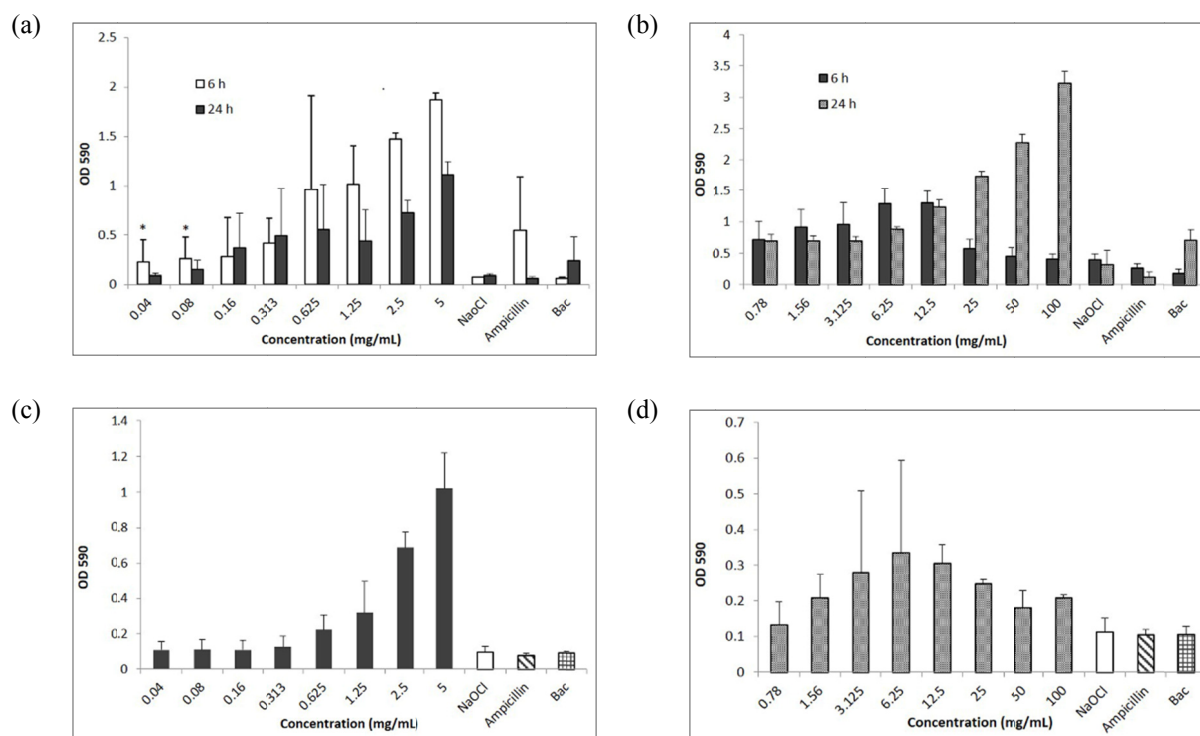

Figure 2. The anti-adhesion and biofilm disruption activities of ginger oil and *O. stamineus* extract on *E. faecalis*. For anti-adhesion, biofilm formation was quantified following 6h and 24 h exposure [(a) ginger oil, (b) *O. stamineus*]. For biofilm disruption activity, the remaining pre-formed biofilm following 24 h of exposure [(c) ginger oil, (d) *O. stamineus*] was quantified

Note. The concentrations the positive controls, NaClO and ampicillin, were 2.5% and 2.5 mg/mL, respectively. Bac: untreated *E. faecalis*, as the negative control. Error bars represent the mean ± SD of two independent experiments performed in triplicates. * Significant differences ($p < 0.05$) between the ginger oil with NaClO.
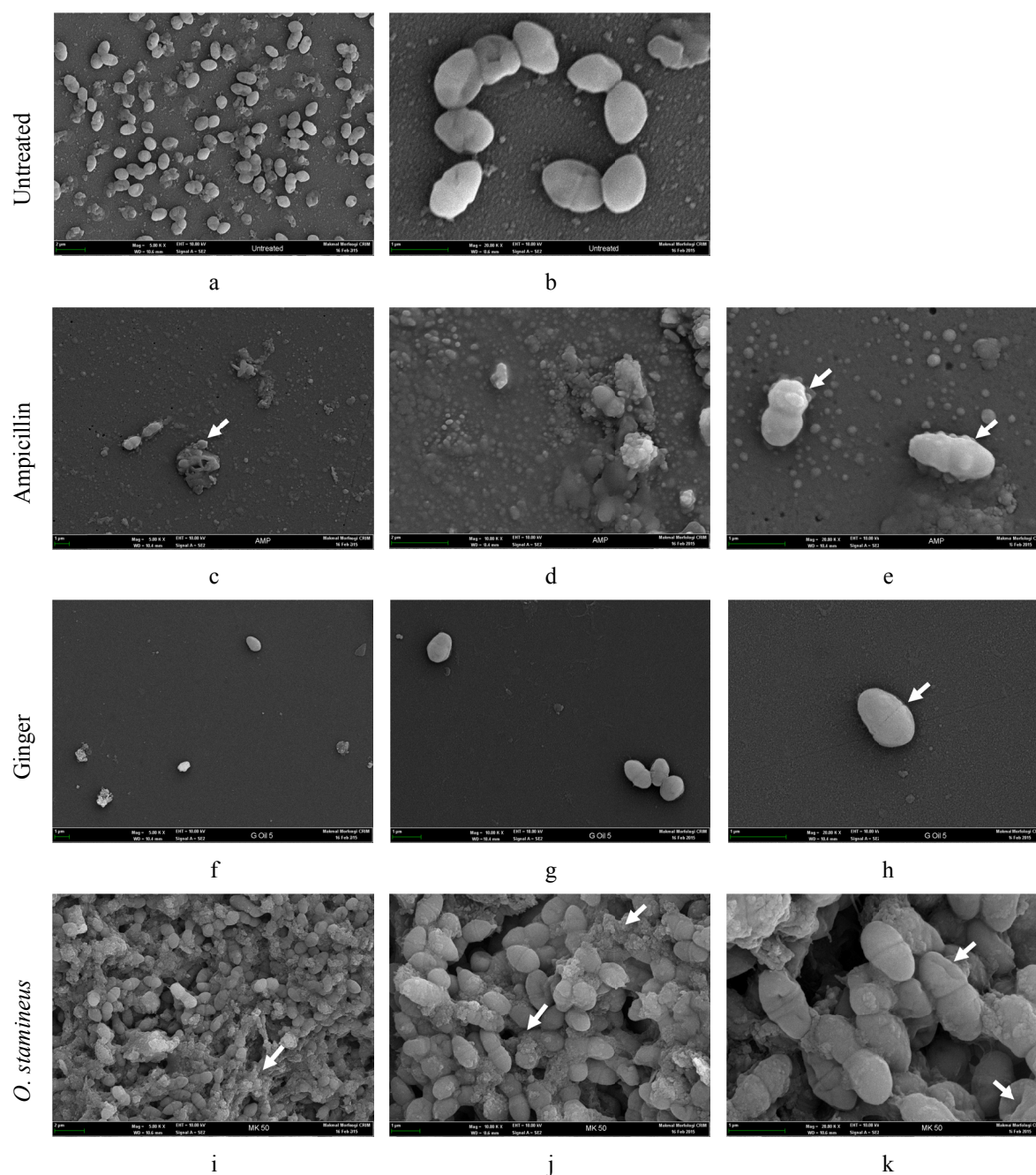
3.3 SEM Analyses

Figure 3. SEM images of the treated *E. faecalis* compared to untreated bacteria and ampicillin

Note. Figure a, c, f, i at 5,000× magnification; Figure b, d, g, j at 10,000× magnification; Figure e, h, k at 20,000× magnification. The tested concentrations of ginger oil and *O. stamineus* were 5 mg/mL and 50 mg/mL, respectively. The ampicillin concentration used was 2.5 mg/mL.

Figure 3a shows attached bacterial cells of the untreated *E. faecalis* where at Figure 3b, the morphology of untreated bacterial cells was seen as ovoid-shaped, with smooth, regular cell surface. There is visibly less *E. faecalis* cell attachment in samples treated with ampicillin (Figures 3c and 3d) compared to untreated sample (Figures 3a and 3b). The cell surface of ampicillin-treated *E. faecalis* is irregular and the shape is elongated with nodular projections (Figure 3e, arrows). Visually, there were less attached cells in ginger oil treated samples (Figures 3f and 3g) compare to untreated samples (Figures 3a and 3b). Irregular *E. faecalis* cell surface was

observed in the ginger oil-treated sample (Figure 3h, arrow). Contrastingly, numerous cells attachment was observed in *O. stamineus*-treated *E. faecalis*, apparently surrounded by particles (white arrowheads) which may have originated from *O. stamineus* extract (Figures 3i and 3j). On higher magnification, the cell surfaces of *E. faecalis* showed irregular, pitted appearance (Figure 3k, arrow).

4. Discussion

E. faecalis are non-spore forming, fermentative, gram-positive organism (Suchitra & Kundabala, 2006). They are usually associated with endodontics infection and implicated more commonly in persistent and secondary infections (Sedgley et al., 2005; Johnson et al., 2006). The persistent infection is caused by microorganisms that can resist intracanal antimicrobial procedures and can survive under extreme conditions (Narayanan & Vaishnavi, 2010). The entry of *E. faecalis* into the root canal usually occurs during endodontic treatment, between appointments or even after root canal treatment (Stuart et al., 2006). *E. faecalis* possesses virulence factors such as lytic enzymes, cytolysin, aggregation substance, and lipoteichoic acid (Stuart et al., 2006). *E. faecalis* is able to form biofilm within the infected root canal system, which renders the bacteria resistant to phagocytosis, antibodies, and antimicrobials (Stuart et al., 2006; Liu 2010). The standard intracanal medicaments used in root canal treatment include calcium hydroxide and disinfectant such as sodium hypochlorite. However, *E. faecalis* biofilm has been reported to resist these common intracanal medicaments (Stuart et al., 2006; Liu, 2010; Sathorn et al., 2007; Haapasalo & Shen, 2012), thus contributing to the failure of root canal treatment and persistent root canal infection. The current irrigant solution, sodium hypochlorite used in cleaning the root canal system is toxic to the surrounding soft tissue (Borzini et al., 2016). Thus, there is a need to find safer, gentler irrigant. *E. faecalis* has been reported to be resistant towards intracanal medicament such as calcium hydroxide when the pH is not maintained at a therapeutic level. This survival capacity of *E. faecalis* is facilitated by a functioning proton pump with the capacity to acidify the cytoplasm whenever the pH is high (Evans et al., 2002). Apart from that, starved *E. faecalis* were able to form a biofilm that was more resistant to 5.25% sodium hypochlorite than those in stationary and exponential phase (Liu, 2010).

Recently, plant materials have been studied widely as medicinal alternatives due to their natural origin, easy availability, efficacy, safety and reportedly lack of microbial resistance (Kamboj, 2000; Rahman et al., 2010). Ginger has been reported to exhibit antibacterial (M. N. J. Gulve & N. D. G. Gulve, 2010; Mohamed et al., 2016), antioxidant (Bellik, 2014), antibiofilm (Kim et al., 2013), anti-gastrointestinal cancer (Prasad & Tyagi, 2015) and antifungal properties (Ficker et al., 2003; Atai et al., 2009). As for *O. stamineus*, studies have reported on the antioxidant, anti-inflammatory (Akowuah et al., 2004; Yam et al., 2008), antifungal (Hossain et al., 2006) and antibacterial properties of this plant (Ho et al., 2010).

The examination of the growth inhibition effect of the studied herbs against *E. faecalis* was carried out using broth microdilution tests. The present study showed that both ginger oil and *O. stamineus* extract exhibited growth inhibition activity towards *E. faecalis* grown as a suspension culture. Comparatively, ginger oil showed a greater inhibitory effect to *O. stamineus* extract. This may be contributed by the herb preparations; ginger was used as essential oil, while *O. stamineus* was prepared by reconstituting the powdered plant with water, thus it can be regarded as a crude preparation of the herb. In the form of essential oil, the antibacterial components of ginger, namely gingerols and shogaol (Park, Bae, & Lee, 2008; Wang et al., 2010) may be in higher concentrations, thus showing good growth inhibition activity. The *O. stamineus* water extract may have lower concentrations of its antibacterial component, rosmarinic acid, which is the primary polyphenol in *O. stamineus* leaf (Akuwoah et al., 2004).

In most oral diseases involving microorganisms, the initiation of a disease requires the pathogens to adhere to the tooth supporting structure or tooth surface (Li et al., 2004). Substances that possess bacterial anti-adhesion activity hold a particular advantage in preventing the adhesion of bacteria to a surface, which is the required initial step for a pathogen to establish infection. In this study, both ginger oil and *O. stamineus* extract showed inverse dose-dependent anti-adhesion activity following both exposure time lengths of 6h and 24 h. Significant anti-adhesion activity relative to ampicillin was observed only at the two lowest ginger oil concentrations. At higher concentrations, more abundant *E. faecalis* adherence was observed for both herbs. It is suggested that there may be unknown compounds in ginger oil and *O. stamineus* extract when at higher concentrations, are able to promote *E. faecalis* adhesion. For *O. stamineus* it has no anti-adhesion activity following 6 h exposure. At 24 h, both studied herbs generally showed lower anti-adhesion activity as the adhered cell layer may have got thicker. No significant biofilm disruption activity was observed for both studied herbs compared to positive control. This may simply because the preformed *E. faecalis* biofilm may have matured, where a high amount of extracellular polymeric matrix that encased the *E. faecalis* cells protects the bacteria from external insults, such as antimicrobial substances (Rickard et al., 2003). *E. faecalis* in suspension mode do not possess this protective

mechanism, which may suggest a greater antibacterial activity of the herbs towards this growth mode than in the biofilm mode.

Our findings suggest that ginger oil, in lower concentrations, has more potential to be developed into antibacterial agent against bacteria in suspension growth mode rather than. The *O. stamineus* extract used in this study was more in a crude form, which may explain its low antibacterial activity. Previous studies which reported the protective effects of *O. stamineus* employed methanolic (Chun-Hoong et al., 2010; Malahubban et al., 2013) and ethanolic extracts (Alshawsh et al., 2012; Mohamed et al., 2012).

The findings on the antibacterial activity of ginger oil in this study were in contrast with the study by M. N. J. Gulve and N. D. G. Gulve (2010) which showed ginger extract has strong anti-bacterial activity against *E. faecalis* in agar diffusion test. Our study reported that ginger oil has a significant antibacterial effect on *E. faecalis* in suspension culture but not in biofilm mode. This may be due to different growth inhibition assays were used, sample size difference and ginger oil concentration difference. Park et al. (2008) showed that ginger extract can inhibit the growth of oral bacteria in suspension mode, which have good agreement with our results. One of the active agents of ginger, [10]-gingerol has been reported to cause damage to the membrane of enterococcus cells (Nagoshi et al., 2006). This damage was similar to the SEM findings in this study, where changes such as pitted cell surface and disintegrated cells were seen on *E. faecalis* following exposure to the studied herbs. According to Lim et al. (2009), hydroethanolic ginger extract exhibits potent antibacterial activity against Gram-positive and Gram-negative bacteria. In the past, essential oils had been demonstrated to be as effective as antimicrobial agents to use in oral care (Tufekci et al., 2008). An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition into and disturb the lipid bilayer of the bacterial cell membrane, rendering them more permeable, leading to leakage of cell contents (Burt, 2004). According to Unlu et al. (2010), cells incubated in essential oil for 48 h showed obvious changes in their morphology and loss of adhesion, which was similar to our findings for *E. faecalis* treated with ginger.

5. Conclusion

Z. officinale and *O. stamineus* showed effective antibacterial effect towards *E. faecalis,* particularly in growth inhibition and anti-adhesion, but less effective in disrupting preformed biofilm. Comparatively, *Z. officinale* oil has more potential to be developed as antibacterial agent against *E. faecalis* in suspension culture compared to *O. stamineus* extract.

Acknowledgements

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