RESEARCH ARTICLE

In vitro inhibitory and biofilm disruptive activities of ginger oil against Enterococcus faecalis [version 1; referees: awaiting peer review]

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Abstract

Background: This study investigated the antibacterial effect of ginger (Zingiber officinale) oil against a common resistant root canal pathogen known as Enterococcus faecalis. The aim of the study was to determine the inhibition of E. faecalis growth in culture suspension and ability to inhibit growth of bacteria through disruption of pre-formed monospecies biofilm.

Methods: Ginger rhizome oil was prepared in two-fold concentration series from 0.04 to 5.00 mg/mL and mixed with brain heart infusion broth inoculated with E. faecalis in anaerobic condition. Among the antibacterial tests performed were the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations using microdilution assays, and anti-biofilm assay on 3-day old pre-form monospecies biofilm on a 94 well-plate. Ampicillin was used as a positive control.

Results: The result showed an in vitro dose-dependent bacteriostatic activity towards E. faecalis in suspension broth (MIC 0.04mg/mL) but no bactericidal activity within the tested concentration range. It was also found that the ginger oil inhibitory activity against E. faecalis was comparably less in anti-biofilm activity than against bacteria cultured in suspension solution.

Conclusion: The study suggests that at determined concentrations, ginger oil has the potential to be used as an antibacterial agent in the management of root canal infections particularly where newly formed E. faecalis is involved.

Keywords
Enterococcus, biofilm, endodontics, Zingiber officinale
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Introduction

Enterococcus faecalis is an opportunistic facultative anaerobe that is well recognised as an oral pathogen associated with persistent apical periodontitis and is highly prevalent in failed root filled teeth. The ability of E. faecalis to enter and survive in root canals, as well as its resistance to root canal medicaments, makes it one of the toughest pathogens to eradicate in endodontics. It has been reported that this pathogen is associated with a high percentage of endodontic failures, which is about one third of the canals of root-filled teeth with persistent periapical lesions. Inevitably, repeated use of calcium hydroxide and sodium hypochlorite as the two most commonly used root canal medicaments and irrigation solution, respectively, has been said to allow E. faecalis to adapt to the sub-lethal environment.

Within the past decade, herbal medicine has begun to offer some beneficial antibacterial activities against oral pathogens. More recently, studies also found the potential antibacterial properties of ginger on oral pathogens, including E. faecalis. In most instances, the effects of ginger extracts were studied on bacteria cultured in vitro and in cell suspensions, using calcium hydroxide or sodium hypochlorite as a control. Limited data is available on the activity of ginger oil in disrupting established E. faecalis biofilm and its comparable effect to antibiotics. Hence, the aim of our study was to explore the in vitro potential of ginger oil as an antibacterial agent against E. faecalis cultured in suspension and biofilm in comparison to ampicillin, as a common antibiotic used for oral infection.

Methods

Extraction and preparation of oil

Ginger oil was prepared from Zingiber officinale Roscoe rhizomes after fresh young ginger were finely sliced, dried and boiled in distilled water for 8h. The oil stock solution (500 mg/mL) was prepared by mixing 100 µL ginger oil with 200 µL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO USA) in an Eppendorf tube. Next, 100 µL of the oil stock solution was pipetted and mixed with 100 µL DMSO in a second tube to produce two-fold dilution at 250 mg/mL. This procedure was repeated six times to produce an eight concentration series of ginger oil solution at 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9 mg/mL respectively. The solutions were vortexed each time after mixing to ensure a thorough mixture were produced. Following this, 20 µL of each oil series were transferred into a 980 µL brain heart infusion (BHI; Oxoid Ltd., Cheshire UK) broth in 2.5 mL universal bottles to produce eight working concentration series of ginger oil mixtures in broth microdilution test at 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0.08 mg/mL respectively. The positive control ampicillin solution was prepared by mixing 500 mg ampicillin powder (Sigma-Aldrich) with 1 mL distilled water, and then eight series of two-fold solutions were prepared similarly to the technique mentioned above.

Culture and maintenance of bacteria

Enterococcus faecalis ATCC29212 (American Type Culture Collection, Virginia USA) used in this study was cultured in BHI (Oxoid Ltd., Cheshire UK) agar plates and regularly maintained in a 37°C incubator with 5% CO₂. Identification and purification of E. faecalis were done through Gram stain test as well as morphology and colony identification. The quantity and viable bacterial number of E. faecalis were determined by colony forming unit (CFU) and standardized with McFarland 0.5 turbidity (1.5 x 10⁸ cfu/mL) prior to the antibacterial assays. Cultures and broths were constantly checked for sterility and contamination. DMSO was used as solvent for ginger oil and ampicillin as the positive control.

Research and ethics approval was obtained from the Universiti Kebangsaan Malaysia Research and Ethics Committee (UKM 1.5.3.5/244/DD/2014/017) for methodology and dissemination of findings.

Broth microdilution assay

An antibacterial assay using the broth microdilution technique was done based on modified NCCLS guideline. A 96-well test plate was divided into two sections where half was inoculated with 50 µL E. faecalis suspension in each well and another section was not inoculated (50 µL broth/well only), as shown in Figure 1, plate rows from A to H. Sample solution series prepared earlier were mixed with the bacterial suspensions in wells and produce a final concentration series of oil mixtures at final concentrations of 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04 mg/mL, respectively. One hundred microliters sample series (ginger oil mixture or ampicillin solution) were dispensed into the prepared 96-well plate. Triplicates were done for each well and test plate. Plates were then incubated at 37°C for 24h. After this, the turbidity of wells on tested plates was measured at 595nm optical density using a microtiter plate reader (Thermo Scientific VARIOSKAN Flash, UK). Bacterial growth inhibition percentage was calculated using the following formula:

\[
\% \text{ Bacterial growth inhibition} = 100\% \left( \frac{\text{mean OD595 (control)} - \text{mean OD595 (test)}}{\text{mean OD595 (control)}} \right) \times 100\%
\]

The reading generated by the device were analysed and the Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of sample that inhibits visible growth of a microorganism after overnight incubation, was determined. Further to this, 20 µL of the mixture from each well that showed bacterial growth inhibition were cultured on BHI agar plates for 24h at 37°C in order to determine whether the inhibition is bacteriostatic or bactericidal in nature. The minimum bactericidal concentration (MBC), defined as the lowest concentration of samples that prevent the total growth of E. faecalis on test plates, was determined.

Anti-biofilm assay

A monospecies biofilm was initially produced by inoculating 200 µL E. faecalis suspension culture at 1.5 x 10⁶ cfu/mL cell density with BHI broth in 96-well plates (modified from Azizan et al). The culture was checked daily for contamination for 4 days and cell density was measured at 595 nm optical density using a microtiter plate reader, and repeated in three separate tests. The findings from this monospecies biofilm development test was charted (Figure 2) and used to determine the age for a stable pre-formed biofilm for the anti-biofilm assay.
In the anti-biofilm assay, *E. faecalis* biofilm was developed and maintained for 3 days. On the third day, culture broth in the plate was pipetted out in slanting position to prevent disruption of biofilm and replaced with 100 µL of fresh broth. Then 100 µL ginger oil or ampicillin solutions at eight respective concentrations were dispensed into the wells and plates were incubated at 37°C for 24h. The turbidity of the bacterial cultures was measured using a microtiter plate reader at 595 nm optical density.

**Statistical analysis**
Each assay was conducted in triplicate and two independent experiments were done. The data collected were analysed with SPSS 21.0. Non-parametric Mann-Whitney test were used
with significant level set as 0.05. Results were accepted as statistically significant if the p value was <0.05.

**Results**
The broth microdilution antibacterial assay showed a dose-dependent inhibition of *E. faecalis* growth when exposed to all tested concentration series of ginger oil solution (Figure 3), but no bactericidal effect of the oil was found within the range tested (0.04 – 5.0 mg/mL). While ampicillin showed almost 80% inhibition for all concentrations tested, ginger oil showed about 76% inhibition at 5.0 mg/mL and 50% and less at 2.50 – 0.04 mg/mL. The difference in means of inhibition between all ginger oil concentrations tested and between ginger oil and ampicillin was found to be statistically significant (p=0.002 and p=0.007, respectively).

In the anti-biofilm test, ginger oil also inhibited the growth of pre-formed biofilm (Figure 4). However, it was found that both ginger oil and ampicillin showed lesser inhibitory effect on pre-formed biofilm than on suspended cells in broth (Figure 3).

![Figure 3. Antibacterial effects of ginger oil and ampicillin against *Enterococcus faecalis* in suspension culture. * significant difference (p<0.05) between ginger oil and ampicillin at 5.00mg/mL.](image)

![Figure 4. Anti-biofilm test of ginger oil and ampicillin against 3-day *Enterococcus faecalis* biofilm.](image)
In this test, the anti-biofilm activity of ginger oil was observed as an increase between 1.25 to 5 mg/mL, yet the activity failed to achieve an acceptable level of inhibition of more than 50%. At 1.25 mg/mL, ginger oil was seen to have somewhat equal biofilm disruptive property as ampicillin, but the means of anti-biofilm activity between the two agents showed no statistically significant difference (p=0.052).

**Discussion**

It has been advocated that ginger, as the stem of *Z. officinale* plant, has strong antimicrobial properties including antibacterial and antifungal against pathogens, including bacteria from the oral cavity. In the present study, it was further discovered that ginger rhizome oil exhibited bacteriostatic activity on *E. faecalis* cultured in plates as monospecies suspension and causes disruption of the pre-formed biofilm. The dose-dependent effect of the oil was accepted as a positive outcome of the study and we predict that with higher concentration (more than 5 mg/mL), ginger oil would have bactericidal effect on *E. faecalis*.

The ability of ginger oil to disrupt 3-day old pre-formed monospecies *E. faecalis* biofilm provided us with a new insight on the antibacterial property of this herbal oil. Although the activity was not as potent as the effect on suspension culture, ginger oil still produced acceptable inhibitory activities against the pathogen in vitro. This study provides clearer appreciation of the *E. faecalis* resistance to antibacterial agents when they are in biofilm form and conforms to other evidence of biofilm resistance.

The use of systemic antibiotics such as ampicillin in acute endodontic infections has raised many concerns over the increase in the prevalence of bacterial resistance in dentistry. On the other hand, the current use of antibacterial irrigation solutions such as sodium hypochlorite, chlorhexidine and iodine as well as calcium hydroxide as canal medicament reduces the needs for systemic antibiotics. However, some degree of toxicity towards vital tissues and allergy reactions to some patients have been reported. Through our study, we found that ginger oil has lower but comparable antibacterial efficacy against *E. faecalis* compared to ampicillin. Further studies to investigate the effects of combined oil-antibiotics may perhaps provide some useful information on the range of its antibacterial activities. Studies have shown that combination of herbal oils with antibiotics does produce adequate synergistic and additive effects against microorganisms.

Lastly, the results of this study demonstrated that at determined concentrations, ginger oil has the potential to be used as an antibacterial agent in the management of failed root canal therapy. Our recent study on the effect of ginger oil on tooth dentine microhardness also provide promising usage of this herbal oil in future endodontics as it offers comparable changes to dentine structure when used as irrigation solution in contrast to sodium hypochlorite and ethylenediaminetetraacetic acid. These two findings may help to access infection deep within the root canal system. Nevertheless, the current clinical scenario does not offer immediate and accurate chairside information on the type of bacterial species involved in specific endodontic infection; hence this finding may not be readily useful for purpose as yet. More studies will be required to evaluate the clinical efficacy of the delivery of ginger oil as an antibacterial agent for endodontic infections.

**Conclusion**

Within the concentration tested (0.04 – 5.0 mg/mL), ginger oil showed an acceptable dose-dependent in vitro inhibitory activity against *E. faecalis* (MIC 0.04mg/mL), while its antibacterial activity towards bacteria in suspension broth was comparatively better than its anti-biofilm activity.

**Data availability**


**Grant information**

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**References**


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