RESEARCH ARTICLE

In vitro inhibitory and biofilm disruptive activities of ginger oil against *Enterococcus faecalis* [version 1; peer review: 1 approved with reservations, 1 not approved]

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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 96 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.

Open Peer Review

Approval Status

	1	2
version 1 28 Nov 2018	view	view

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Any reports and responses or comments on the article can be found at the end of the article.

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 teeth¹. 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 endodontics²⁻⁷. 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 lesions⁸. 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 environment⁹.

Within the past decade, herbal medicine has begun to offer some beneficial antibacterial activities against oral pathogens^{10,11}. More recently, studies also found the potential antibacterial properties of ginger on oral pathogens, including *E. faecalis*¹²⁻¹⁶. 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 8h¹⁷. The oil stock solution (500 mg/mL) was prepared by mixing 100 μ L ginger oil with 200 μ L dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO USA) in an Eppendorf tube. Next, 100 μ L of the oil stock solution was pipetted and mixed with 100 μ L 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 μ L of each oil series were transferred into a 980 μ L 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 guideline¹⁸. A 96-well test plate was divided into two sections where half was inoculated with 50 μ L *E. faecalis* suspension in each well and another section was not inoculated (50 μ L 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 respectively¹⁹. 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:

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

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 micro-organism after overnight incubation, was determined. Further to this, 20 μ L 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 plates²⁰, was determined.

Anti-biofilm assay

A monospecies biofilm was initially produced by inoculating 200 μ L *E. faecalis* suspension culture at 1.5 x 10⁸ cfu/mL cell density with BHI broth in 96-well plates (modified from Azizan *et al*¹⁹). 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.

Figure 1. Antibacterial assay plate layout for minimum inhibitory concentrations and anti-biofilm test.

Figure 2. *Enterococcus faecalis* biofilm development test.

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 μ L of fresh broth. Then 100 μ L 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.

Figure 4. Anti-biofilm test of ginger oil and ampicillin against 3-day *Enterococcus faecalis* biofilm.

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 ($p=0.052$).

Dataset 1. File containing raw data for biofilm development test, anti-biofilm assay and suspension assay

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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^{21–27}. 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^{28–30}.

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^{31–33}. 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^{34–36}. 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 microorganisms³⁷.

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 acid³⁸. 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

F1000Research: Dataset 1. File containing raw data for biofilm development test, anti-biofilm assay and suspension assay. , <https://doi.org/10.5256/f1000research.16851.d225122>³⁹

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Open Peer Review

Current Peer Review Status: ? ✖

Version 1

Reviewer Report 13 June 2019

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The study aimed to assess the antibacterial and antibiofilm properties of ginger oil on *E. faecalis* which is a pathogen often isolated from tooth root canals. *E. faecalis* used were in the planktonic (cell suspension) and monospecies biofilm forms. Tests performed include MIC, MBC and an antibiofilm assay.

Comments:

1. Introduction: Adequate

2. Methods:

- (i) OD readings from ELISA plate reader were used as a measurement of cell density in both the planktonic and biofilm state. This procedure should be revised as in the former, the cells are free in the suspension while in the later, the cells are sedimented/attached to the bottom base, which blocks vertical light source from the bottom and may contribute to higher OD readings. A different set of readings would be obtained if using a spectrophotometer with horizontal light source.
- (ii) An inoculation of 20ul of the broth mixture on agar plates would not be able to indicate a bacteriostatic effect of a test agent, it can only confirm the bactericidal activity of a test agent i.e. when there is no colony growth.
- (iii) How was a stable 3-day preformed biofilm defined, and how can Fig. 2 explain this?
- (iv) The 3-day biofilm could have resulted from sedimentation since the biofilm was left static for 3 days. It is thus suggested that the biofilm is gently washed first before adding the test agent. The use of a shaking incubator is also suggested.

3. Results:

- (i) What were the MIC and MBC for ginger and ampicillin towards *E. faecalis*?
- (ii) Figure 3 does not really show a dose-dependent antibacterial response of *E. faecalis* to ginger oil, or to ampicillin. This would be obvious if error bars were shown in the figures.

(iii) It is not accurate to claim that the anti-biofilm activity of ginger oil was observed to increase between 1.25 to 5 mg/ml. referring to Fig. 4 the increase were insignificant, and again this would be obvious if the error bars were shown.

4. Discussion:

Cross referencing to more relevant journals/works to defend and justify the study outcome would help improve the discussion.

Overall comment:

The manuscript was poorly prepared and certain details such as error bars in the figures were missing. Many steps were over simplified. Analysis and presentation of results can be further improved to give meaningful outcome. I agree with Reviewer 1 that the conclusion was rather over-interpreted, and very weak considering only one species of bacterium was used. More experiments are required to come to such conclusion.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral environment, Oral biofilm, Antimicrobial agent

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 16 January 2019

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In this study, the authors assessed the inhibitory activities of ginger oil in comparison to ampicillin against *Enterococcus faecalis*, both against planktonic and biofilm cultures, with the clinical background of analyzing novel treatment ideas for root canal infections caused by *E. faecalis*. Ginger oil has been shown to exhibit antibacterial effects against *E. faecalis* in some *in vitro* studies, but its anti-biofilm activity has not been investigated yet.

Although the authors came up with an in principle adequate experimental set-up, the main criticism concerns the fact that only one laboratory strain has been analyzed (ATCC 29212), but no clinical isolates recovered from root canal infections. Clinical isolates usually show a different behavior than laboratory strains concerning antimicrobial susceptibility patterns, making it absolutely necessary to include an adequate number of clinical isolates when drawing conclusion for therapeutic treatments of infections. Further, it remains questionable whether the analyzed concentrations of ginger oil apply as therapeutic dosages. The lowest concentration (range 40 mg/L to 5000 mg/L) analyzed for both ginger oil and ampicillin, the reference substance in this study, is still 40 times higher than the MIC of ampicillin of 1 mg/L for *E. faecalis* ATCC 29212¹. Unfortunately, the conclusion drawn from the presented results is in our opinion over-interpreted (mainly due to n=1) and not conclusive from the data presented. As the authors state in the result part, the biofilm inhibitory activity of ginger oil was not strong enough to make it a significant result (quote “activity failed to achieve an acceptable level of inhibition of more than 50%”), contradicting very much the conclusion of the discussion part (quote “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*”). Ampicillin, a common antibiotic for oral infections and the reference substance, showed stronger effects than ginger oil at all concentrations tested in both of the planktonic and biofilm experiments!

Regarding the language, only the sentences in which the meaning was not clear were marked and suggestions for improvement were made. Typos, wrong choices of words and sentence constructions were neglected.

Introduction: approved

Methods: approved with reservations/not approved due to n=1

Results: approved

Discussion: not approved

Major comments:

1. Methods: Please include at least three root canal *E. faecalis* isolates in your study. Further, please state some data/references about therapeutic doses of ginger oil to better understand the suitability of the tested concentrations for therapeutic treatment.
2. Method anti-biofilm assay: We are struggling with your method of choice (OD

measurement) for the establishment of the biofilm growth. Firstly, a measurement of the OD value rather assesses the planktonic but not the biofilm state of the bacteria and further does not discriminate between viable and dead cells. We recommend performing a CFU determination of the detached biofilms at day 1, 2, 3 and 4 of biofilm growth² to determine the viable cell number. This will tell you how the OD measurement correlates with cell numbers. Also, you might want to consider changing the medium every 24hr to enhance biofilm formation. Secondly, we do not understand why you have chosen a 3-day old biofilm for your subsequent anti-biofilm assay as the highest OD value is observed on day 1 (see Figure 2). Please explain your choice. For the anti-biofilm assay, please also verify for at least one strain that lower OD values (compared to the control) correlate with lower cell numbers and therefore stronger inhibitory effects of ginger oil.

3. Results Bactericidal effects (MBC): If you plated the samples on agar plates to determine the MBC as described in the method section, it would be interesting to see the CFU numbers to see the correlation between OD values and viable bacteria (see above)!
4. Discussion: Disruption is the wrong word because you were analyzing biofilm growth inhibitory, but not biofilm eradication effects! Also, "we found that ginger oil has lower but comparable antibacterial efficacy compared to ampicillin" is a very contradicting statement and in terms of your results wrong. Ampicillin showed stronger effects than ginger oil at all concentrations tested in both of the planktonic and biofilm experiments!
5. Conclusion: Why do you state 0.04 mg/mL as the MIC of ginger oil for ATCC 29212? At this concentration, only approximately 5% growth inhibition was observed (Fig. 3).

Minor comments:

1. Abstract Methods: You state that *E. faecalis* was grown in anaerobic conditions but this is not described in the method part of the main paper. For MIC testing according to CLSI guidelines, enterococci are grown aerobically (so at 37°C and 5 % CO₂ as you stated correctly in the method section).
2. Abstract methods: Please consider revision of the sentence: "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 96 well-plate." E.g. "Among the antibacterial tests performed were the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) microdilution assay as well as an anti-biofilm assay on 3-day old pre-formed biofilms on 96 well-plates."
Further, it would be helpful for the reader to clarify which parameter was assessed in your anti-biofilm assay. If I understood your experiments correctly, you assessed the so-called minimum biofilm inhibitory concentration (MBIC³) in contrast to biofilm eradication effects (aka MBEC).
3. Abstract results: Please consider revision of the sentence. "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." E.g. "It was also found that the inhibitory activity of ginger oil was lower in biofilm cultures compared to planktonic cultures of *E. faecalis*."
4. Introduction: Your introduction is precise and on point. Maybe you could add 1-2 sentences about the definition/meaning and clinical problem of biofilm formation.
5. Introduction: First paragraph, last sentence: "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". Please re-write this sentence as I do not get the meaning. Do you mean *E. faecalis* is tolerant to sodium hypochloride and calcium hydroxide treatment? Also, please change "Within the past decade, research has begun to explore some beneficial antibacterial activities of herbal medicine against oral pathogens."

6. Methods: Please state the source of the ginger root as the quality and therefore the reproducibility of the experiments might vary depending on the growth conditions.
7. Method, part culture and maintenance of bacteria: The last sentence (DMSO was used as solvent for ginger oil...) is redundant and does not fit in this paragraph.
8. Method, part broth microdilution assay: This sentence is rather confusing because the two sections you describe are not visible in Figure 1. Please clarify your pipetting scheme.
9. Results: Please make the captions below the figures more informative by including more details about how the results presented in the figure were created (e.g. Figure 2: which strain is shown, how were the biofilms grown,...).

References

1. EUCAST: EUCAST Quality Control. [accessed 15th Jan 2019]. [Reference Source](#)
2. Thieme L, Klinger-Strobel M, Hartung A, Stein C, et al.: In vitro synergism and anti-biofilm activity of ampicillin, gentamicin, ceftaroline and ceftriaxone against *Enterococcus faecalis*. *Antimicrob Chemother.* 2018; **73** (6): 1553-1561 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Macià MD, Rojo-Molinero E, Oliver A: Antimicrobial susceptibility testing in biofilm-growing bacteria. *Clin Microbiol Infect.* 2014; **20** (10): 981-90 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: infectious diseases, clinical microbiology, antibiotic resistant bacteria, biofilms, enterococci,

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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