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RESEARCH ARTICLE

Chemical compounds Anti-bacterial of *Citrus aurantifolia* Ethanol Extract to Inhibit the Early Biofilm Formation and Growth of *Enterococcus faecalis* Root Canal Isolate

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ABSTRACT:

Enterococcus faecalis (*E. faecalis*) is a gram-positive commensal bacterium involved in the pathogenesis of dental root canal infections. *Citrus aurantifolia* can inhibit the adhesion and growth of *E. faecalis*. The study aims to evaluate the Antibacterial properties of *Citrus aurantifolia* ethanol extract to Inhibit the early biofilm formation and growth of *Enterococcus faecalis* Root Canal Isolate. The necrotic tissue of the root canal from infected patients was cultured by *E. faecalis* and *E. faecalis* ATCC 29212 (bacteria control) on ChromAgar medium. The inhibition of *E. faecalis* biofilm was measured using a violet crystal assay. In contrast, confirmation of *E. faecalis* growth from planktonic and biofilm masses was tested using the Total plate count assay, and data were analyzed using the Kruskal-Wallis test. All concentrations of ethanol extract of *Citrus aurantifolia* showed their potential in inhibiting early phase (18 hours) biofilm formation and growth of *E. faecalis* clinical isolates and ATCC 29212 from planktonic and biofilm masses in vitro. At concentrations 0.5, 2, 6, and 12% have a potent biofilm inhibition, and concentrations of 14% and 16% are moderate ($p > 0.05; 0.091$). Extracts with concentrations of 0.5, 2, and 6% were bacteriostatic against the clinical isolate of *E. faecalis*, and ATCC isolates from planktonic, while concentrations of 12, 14, and 16% were bactericidal ($p > 0.05; 0.548$). Specifically, *E. faecalis* clinical isolates from biofilm masses were only bacteriostatic ($p < 0.05; 0.01$). The inhibition of the biofilm from both *E. faecalis* and the growth of the colony was not significantly different ($p > 0.05; 0.753$) and does not positively correlate ($r = -0.095$). *Citrus aurantifolia* has more potent biofilm inhibition against *E. faecalis* ATCC 29212 (100%) than *E. faecalis* clinical isolate (75%). The bacteriostatic and bacteriocidal properties of extracts against the *E. faecalis* from planktonic are more susceptible than those from biofilm mass sources.

KEYWORDS: Biofilm, *Citrus aurantifolia*, *Enterococcus faecalis*, Growth, Root Canal.

INTRODUCTION:

Enterococcus faecalis (*E. faecalis*) is a microorganism detected in persistent endodontic infections. The prevalence ranges from 30% to 80% with various forms of periradicular disease, including primary endodontic conditions associated with asymptomatic chronic periradicular lesions¹. In prior endodontic infection cases, *E. faecalis* is found in about 4 to 40% and is the only microorganism in the dental root canal because it has a higher tendency to form a biofilm¹, thus increasing

the growth of *E. faecalis* cells as one of the strategies of this bacterium to resist antibiotics². This phenomenon is related to various survival factors and virulence of *E. faecalis*.

Stuar (2006) reports that as a defense strategy, *E. faecalis* increases competition with other microorganisms in the root canal, takes calcium in the dentinal tubules, dissolves oxygen, prevents dentinal tubulin repair, and forms a smear layer on the surface of the tubules to prevent nutrient supply³. These phenotypic properties are facilitated by certain virulence factors, including lytic enzymes, cytolysin, aggregation agents, pheromones, and lipoteichoic acid. These properties prevent other bacterial cells from growing in the root canal⁴. Estrela (2009) reported that *E. faecalis* has serine protease, gelatinase, and collagen-binding protein (Ace), facilitating interactions in the dentinal tubules while suppressing lymphocyte action in endodontic treatment⁵. Besides, *E. faecalis* can form biofilms that protect themselves from damage by allowing 1000 times more resistance to phagocytosis, antibodies, and antimicrobials than non-biofilm bacteria.⁶

The use of Chlorhexidine 2% combined with sodium hypochlorite is helpful to prevent the development of *E. faecalis* in the dental root canal system⁷. However, the two antiseptics do not wholly eliminate *E. faecalis*, even though 10% calcium hydroxide has been added⁸. This phenomenon shows that the *E. faecalis* in the root canal is very difficult to be eradicated concerning the increase in biofilm formation by *E. faecalis*, which leads to resistance⁹. Therefore, *E. faecalis* biofilm is considered an appropriate model for testing new antimicrobial treatments. One of the main goals of root canal treatment is to eliminate bacteria from the root canal system to treat or prevent apical periodontitis.

Lebeaux (2014) reported that the bacteria in the biofilm community are not only up to 1000 times more resistant to antimicrobial agents and antibiotics than planktonic bacteria (free-living), but also avoid the immune system effectively¹⁰. Hence, bacterial biofilm *E. faecalis* is a significant barrier to endodontic disinfection in the root canal system. Therefore, methods to promote the spread of biofilms can ultimately improve treatment outcomes. The main concern for preventing intra-canal biofilms is antimicrobial irrigation solutions during root canal treatment. However, the most commonly used antimicrobial irrigation solution has a limited ability to remove biofilms from the root canal altogether, even causing persistent infections¹¹. Thus, developing new anti-biofilm anti-biofilm agents to achieve effective and predictable disinfection from the root canal system.

Natural plants such as *Citrus aurantifolia* have the potential as antibacterial. Mubarak (2018) reported that *Citrus aurantifolia* has an anti-adhesion effect in an acidogenic atmosphere¹². Nevertheless, according to Bolhari (2012), *Citrus aurantifolia* mixture in alcohol cannot effectively remove the smear layer compared to 17% EDTA during root canal therapy¹³. In general, it is challenging to eliminate the smear layer in the root canal. It is also exacerbated by planktonic frequencies and biofilm masses promoting the interaction between the root canal surface protein and chemical substances in antiseptic material^{9,14}. So it can be assumed that the prevention of planktonic phase and biofilm masses by *Citrus aurantifolia* can reduce the growth of *E. faecalis*.

MATERIAL AND METHODS:

This research has passed No. Ethical Clearance 29 Ethical Approval/FKGUI/V/2018, issued by the Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia. The study sample was *E. faecalis* bacteria isolated from volunteers who experienced endodontic infections from the Hospital RSGMP-FKGUI Jakarta Indonesia. Bacteria *E. faecalis* isolate ATCC 29212 was used as a comparison. Meanwhile, the test material used in this study was ethanol extract of *Citrus aurantifolia* peel with concentrations of 0.5, 2, 6, 12, 14, and 16%, and Chlorhexidine (CHX), the gold standard in the treatment of root canal infection, was used as the positive control.

Plant Material:

Lime (*Citrus aurantifolia*) was obtained from Sukalarang, Kec. Sukalarang, Sukabumi Regency, Jawa Barat 43191 Bandung Jawa Barat, Indonesia. GPS Coordinates 6°52'38.0"S 107°00'33.8"E and identified by one of the authors (Nur Asmah). The voucher specimen was deposited at the Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.

GC-MS Analysis:

The GC-MS analysis of *Citrus aurantifolia* ethanol extract was performed at the Provincial Health Laboratory of Special Capital of Jakarta using a Shimadzu QP2010PLUS system equipped with an AOC-20i auto-sampler, and the Gas Chromatograph was coupled to a Mass Spectrometer equipped with an Elite-5MS fused a capillary column of 300.25 m ID0.25 m df. With an ionization energy of 60 eV, the electron ionization system was operated in electron impact mode. Helium gas was used as a carrier at a 1mL/min constant flow rate and a volume injection.

Isolation and Culture of *Enterococcus faecalis*:

Research subjects were given an explanation of the situation during the treatment process. After the patients agreed, they filled out an informed consent sheet. The

diagnosis was made according to pulp formation with periapical radiolucency but without subjective complaints. The selected sample teeth were then polished with pumice and installed in the rubber dam. The tooth surface was then cleaned with H₂O₂ (Sigma Chemical, UK) and sterilized with 2.5% sodium hypochlorite (Sigma) for 30 sec. After the caries was cleaned, the cavity was fixed with a cotton pellet. Access preparation was carried out with round and blunt diamonds without water cooling. Scoping of the root canal was done without irrigation solution using K file needles ranging from No. 10 to 15 (Dentsply, Maillefer, Ballaigues, Switzerland). Bacterial samples from the root canal were taken with file no 20. The file was inserted into the root canal in a filling motion, minus 1mm from the apex according to the length of work that appears on the apex locator. Then saline was dropped, and two paper points were attached to the root canal with a predetermined distance for 60 seconds. The paper points (Dentsply, Maillefer, Ballaigues, Switzerland) and K files were then attached to Eppendorf tubes containing 500 μ L Phosphate-buffered saline (PBS, Sigma Chemical Company, St. Louis, MO, USA), followed by vortexing, then 50 μ L of the bacterial sample solution was dropped into a medium selective Chrom Agar (ChroMagar, Springfield, NJ 07081, USA), stoppered and then put into an anaerobic jar (Anaerogen, Oxoid, Hampshire, UK) and incubated at 37°C for 48 h. Bacterial growth on the chromium agar was identified (bluish-green showing *E. faecalis* colony), then biomolecular confirmation using conventional PCR based on Bachtiar's working principle (2015)¹⁵. Furthermore, *E. faecalis* was cultured in a medium of brain heart infusion broth (BHI) liquid made as stock by mixing 50% glycerol, stored at -80°C. *E. faecalis* bacteria were compared with colony density based on optical density (0.08-0.1nm), equivalent to <300 CFU/mL¹⁶.

Biofilm Assay:

Biofilm test with violet crystals refers to Gani (2007)¹⁷. A total of 100 μ L *E. faecalis* clinical isolate and ATCC 29212 from a balanced BHI solution were placed into the well and incubated for 3 hours at 37°C, the surface part, which is a BHI solution was removed, and the test extract with a concentration of 0.5, 2, 6, 12, 14, 16% (30 μ L: 70 μ L) was gently added into the well and subsequently incubated at 37°C for 18 h. The supernatant was then discarded and rinsed with 100 μ L PBS. Later, 100 μ L of 1% violet crystal was introduced into the well and incubated at room temperature (28°C) for 15 min. The favorable interaction of biofilm masses with violet crystals is characterized by the union of violet crystal blue color with bacterial cells at the base of the plate. The violet crystal solution in the well is removed, and the bacterial cells are washed with PBS 2 times. Then each vessel was rinsed with 96% alcohol.

Then the results were read with an Elisa reader spectrophotometer at a wavelength of 450nm. Measurements on the ELISA reader-generated absorbance values expressed as Optical Density (OD).

Total Plate Count Assay:

The total plate count method refers to Bachtiar (2016).¹⁸ *E. faecalis* bacteria that have been exposed by test material for 18 hours were diluted to the factor of 10⁴. Planktonic bacteria was then taken from the well and transferred to a 1.5mL Eppendorf tube containing PBS. Furthermore, 10 μ L PBS containing *E. faecalis* planktonic was cultured on BHI agar. After the planktonic was removed the plate was washed with 100 μ L PBS, then the PBS was discarded, then 100 μ L of the solution was poured into well plate 96, then biofilms at the bottom of the well were dredged. Then it was put into a tube to make 104 dilutions, and 10 μ L of a solution containing *E. faecalis* biofilm was cultured on BHI agar. Both plates were incubated for 24 h at 37°C. The growing colonies were counted. Colonies <300 CFU/mL are considered bacteriostatic, whereas not growing colonies are considered bacteriocidal. The effect of the concentration of *Citrus aurantifolia* extract on the growth of *E. faecalis* colonies was determined based on the bacterial growth phase (lag phase (Adjustment), Logarithmic /exponential phase, stationary phase, and death phase.

Statistical Analysis:

Data on *E. faecalis* biofilm formation and colony growth originating from planktonic and biofilm masses were analyzed by the Kruskal-Wallis test with a significance level of p<0.05 and Spearman correlation (r = 1) was a strong relationship.

RESULTS AND DISCUSSION:

Fig 1. Shown that the caryophyllene, bicyclogermacrene, delta-elemene, beta-elemene, and gamma-elemene compounds are known to have antibacterial and anticancer properties because they have solid cytotoxic properties against bacteria cells and human colorectal cancer cells. Recent studies have shown that cytotoxicity induced by β -caryophyllene and caryophyllene are related to its apoptotic nature through DNA fragmentation and mitochondrial pathways¹⁹.

Fig 2 shows that the lower graph indicates better inhibition. In general, all concentrations could inhibit the formation of *E. faecalis* biofilms based on positive control (CHX). Based on the biofilm inhibition scale, the concentrations of 0.5%, 2%, 6%, and 12% have a potent inhibition about CHX (strong). At the same time, 14% and 16% concentrations have moderate inhibition (Table 1). Based on Kruskal Wallis analysis, it can be explained that there were no significant differences in the

inhibition of Biofilm between *E. faecalis* clinical isolates and ATCC 29212 isolates ($p > 0.05$; 0.091). There was also no significant difference ($p > 0.05$; 0.390) based on the concentration. Thus, it can be assumed that ethanol extract of *Citrus aurantifolia* has the same effect of inhibiting *E. faecalis* biofilm from both clinical isolates and ATCC isolates, and the inhibition was not influenced by concentrations in the early phase (18 h) on biofilm formation and colony growth in planktonic and biofilm masses

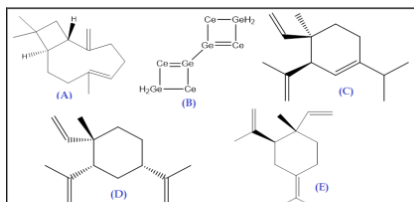


Fig. 1: Antibacterial compound of *Citrus aurantifolia* ethanol extract. The result of GCMS analysis found five chemical compounds that have antibacterial potential. (a) caryophyllene (b) bicyclogermacrene, (c) delta-elemene, (c) beta-elemene, dan (e) gamma-elemene.

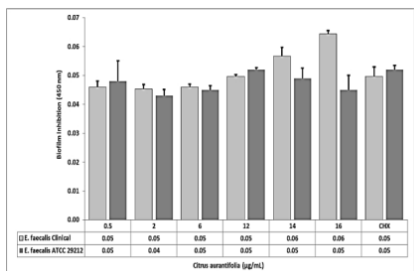


Fig. 2: Inhibition biofilms by ethanol extracts of *Citrus aurantifolia* with various concentrations. The strongest inhibition was found at concentrations of 0.5%, 2%, 6%, and 12%. While medium inhibition (moderate) was found at 14% and 16% concentrations based on positive control (CHX). CHX has a better response to clinical *E. faecalis* isolate than ATCC 29212 isolates. Bars (Biofilm inhibition) and Bar errors (Standard Deviation)

Fig 3 and Fig 4 shows that the growth of *E. faecalis* from both isolates originating from planktonic and biofilm masses was determined by the concentration of the *Citrus aurantifolia* extract. *Citrus aurantifolia* showed bacteriostatic properties at lower concentrations of 0.5, 2, and 6% and more bactericidal at the highest 12, 14, and 16% concentrations. This property correlates with the activity of the test material based on the origin of *E. faecalis* (planktonic and biofilm masses). Specifically, *Citrus aurantifolia* extract has a poor inhibition ability

against *E. faecalis* clinical isolate (bacteriostatic) sourced from mass biofilm compared to *E. faecalis* isolate ATCC. So it can be assumed that the bacteriostatic and bactericidal activity of both *E. faecalis* isolates is influenced by the concentration and environmental changes based on the intensity of the bacterial response.

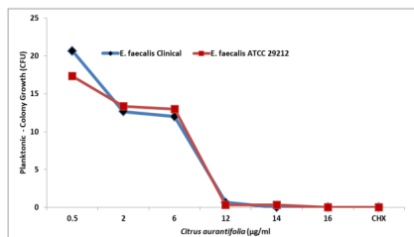


Fig. 3: Growth of *E. faecalis* colonies in planktonic. At concentrations of 12, 14, and 16%, the *Citrus aurantifolia* extract was bactericidal against *E. faecalis*, both isolates. Whereas concentrations of 0.5, 2, and 6% were bacteriostatic against *E. faecalis*, both isolates.

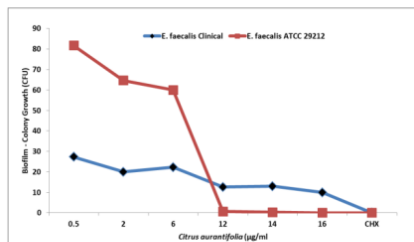


Fig.4: Growth of *E. faecalis* colonies biofilm masses. *Citrus aurantifolia* extract was only bacteriostatic against *E. faecalis* clinical isolates, whereas against *E. faecalis* ATCC 29212 was bactericidal in concentrations of 12, 14, and 16%) and concentrations of 0.5, 2, and 6% were bacteriostatic.

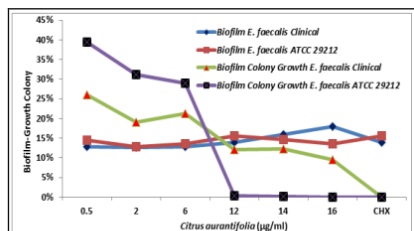


Fig. 5: Relationship of inhibition of *E. faecalis* Biofilm by *Citrus aurantifolia* extract to the viability of *E. faecalis* cells in colonies sourced from biofilm masses. *Citrus aurantifolia* extract could accelerate the phase lag, stationary phase, and death phase to prevent biofilm formation by *E. faecalis* clinical isolates and ATCC 29212 isolates. The inhibitory stage of biofilm formation is in line with the bacterial growth phase

Fig 5 shows that the frequency of inhibition of *E. faecalis* biofilm clinical isolates is in line with the log phase (growth) of *E. faecalis* which adjusts to environmental changes or adaptation (lag phase) to the influence of the test material (0.5, 2, and 6%). A 12% concentration is a stationary phase (without a death phase), where bacteria begin to decrease growing due to a decline in productivity indicated by a stable inhibitory power (Table 1). While the frequency of inhibition of *E. faecalis* Biofilm ATCC 29212 isolates correlated with the lag of the bacterial phase at a concentration of 6%. The stationary phase possibly occurs between levels of 6% and 12%. While the death phase occurs more quickly

due to the influence of test material starting from 12, 14, and 16% concentrations.

Table 1 shows that the *Citrus aurantifolia* extract has a more potent biofilm inhibition against *E. faecalis* isolate ATCC (100%) compared to clinical *E. faecalis* isolate (66%) and moderate (34%). The inhibition of biofilm formation by these two bacteria was not significantly different ($p > 0.05$). Meanwhile, *Citrus aurantifolia* extract is bacteriostatic and bactericidal against *E. faecalis* from planktonic and mass biofilm. In general, *Citrus aurantifolia* extract has a more substantial effect on *E. faecalis* clinical isolates and ATCC

Table 1: Distribution and frequency of antibacterial of *Citrus aurantifolia* to the inhibitory of biofilm formation and growth of *E. faecalis*

Virulence Assay	Material Assay (%)	<i>E. faecalis</i> Clinical			<i>E. faecalis</i> ATCC 29212			P	Description
		Value	Freq (%)	Scale	Value	Freq (%)	Scale		
Biofilm Formation (450 nm)	0.5%	0.05	13%	Strong	0.05	14%	Strong	$P > 0.05$	<i>Citrus aurantifolia</i> effects on the biofilm formation of <i>E. faecalis</i> Clinical; Strong (66%) and Moderate (34%). <i>E. faecalis</i> ATCC 29212; strong (100%)
	2%	0.05	13%	Strong	0.04	13%	Strong		
	6%	0.05	13%	Strong	0.05	13%	Strong		
	12%	0.05	14%	Strong	0.05	16%	Strong		
	14%	0.06	16%	Moderate	0.05	15%	Strong		
	16%	0.06	18%	Moderate	0.05	13%	Strong		
	CHX	0.05	14%	Strong	0.05	16%	Strong		
Planktonic -Colony Growth (CFU/ml)	0.5%	21	45%	Bacteriostatic	17	39%	Bacteriostatic	$P > 0.05$	<i>Citrus aurantifolia</i> effects on the Planktonic Cell Growth of <i>E. faecalis</i> Clinical; Bacteriostatic (25%) and Bactericidal (75%). <i>E. faecalis</i> ATCC 29212; Bacteriostatic (33%), Bactericidal (67%)
	2%	13	28%	Bacteriostatic	13	30%	Bacteriostatic		
	6%	12	26%	Bacteriostatic	13	29%	Bacteriostatic		
	12%	1	1%	Bacteriostatic	0	1%	Bactericidal		
	14%	0	0%	Bactericidal	0	1%	Bactericidal		
	16%	0	0%	Bactericidal	0	0%	Bactericidal		
	CHX	0	0%	Bactericidal	0	0%	Bactericidal		
Biofilm - Colony Growth (CFU/ml)	0.5%	27	26%	Bacteriostatic	82	39%	Bacteriostatic	$P < 0.05$	<i>Citrus aurantifolia</i> affects the Planktonic Cell Growth of <i>E. faecalis</i> Clinical; Bacteriostatic (100%). <i>E. faecalis</i> ATCC 29212; Bacteriostatic (33%), Bactericidal (67%)
	2%	20	19%	Bacteriostatic	65	31%	Bacteriostatic		
	6%	22	21%	Bacteriostatic	60	29%	Bacteriostatic		
	12%	13	12%	Bacteriostatic	1	0%	Bactericidal		
	14%	13	12%	Bacteriostatic	0	0%	Bactericidal		
	16%	10	9%	Bacteriostatic	0	0%	Bactericidal		
	CHX	0	0%	Bactericidal	0	0%	Bactericidal		

This study examines the role of *Citrus aurantifolia* ethanol extract in inhibiting the formation of *E. faecalis* biofilm in the early phase and confirming the growth of *E. faecalis* colonies from the planktonic origin (free) with sources of biofilm masses. Planktonic and bacterial biofilms are the basis of the lag and log phase of bacterial growth before invading and infecting host cells²⁰. The results revealed that the extract of *Citrus aurantifolia* inhibited the early phase of the *E. faecalis* biofilm formation (18 hours). The growth of colonies in the planktonic phase and the formation phase of bacteriostatic and bactericidal biofilms.

Fig 1 shows the chemical compound of *Citrus aurantifolia* as anti-bacteria and oxidants. The compound of caryophyllene with lime peel extracts, including beta-elemene and delta-elemene,

bicyclogermacrene also has antimicrobial properties. Costa (2008) Reported that the compound bicyclogermacrene, delta elemene, beta- elemene, and gamma- elemene able to inhibit the growth of bacteria gram-positive *Propionibacterium acnes* and *Staphylococcus aureus*, but also capable of inhibiting the rate of growth of the fungus *Malassezia furfur*²¹. The test proves that both elemene compounds have either antibacterial and anti-fungal activities. Unlike elemene compounds and bicyclogermacrene, unlike elemenes, bicyclogermacrene shows unique properties as anti-infectious compounds and can accelerate wound healing. Together with these germanesene compounds, several other compounds found in the essential oils obtained from various plants can become active compounds for infections of various diseases and accelerate new cell formation²².

Fig 2 shows that the ethanol extract of *Citrus aurantifolia* has a high inhibitory power at low concentrations. The inhibition of *E. faecalis* ATCC 29212 biofilms is stronger than that of *E. faecalis* Clinical. These results indicate that clinical *E. faecalis* is a bacterium that has previously been exposed to many antiseptic drugs when treating dental root canals²³, the possible level of resistance to anti-bacteria is highly correlated with the results of this study. Whereas *E. faecalis* ATCC 29212 is a laboratory isolate used as a control, where it is still pure and has never been exposed to antiseptic, it has a very high susceptibility to antiseptic. It is correlated with the results of this study.

The anti-biophilic power shown by *Citrus aurantifolia* correlates with the antibacterial compounds that consisted in the extract like caryophyllene, d-limonene, alpha-tocopherol, citropen, and phytol based on the results of the Gas Chromatography-Mass Spectrometry (GC-MS) examination, which is part of this research. These three compounds are reported to inhibit the development of bacteria during the pathogenesis of infection, especially in the formation of biofilms, and inhibit growth. In vitro modeling, in inhibiting biofilms in the early phase, *Citrus aurantifolia* works by suppressing the log phase and stationary phase of *E. faecalis*.²⁴ Besides, *Citrus aurantifolia* is associated with the release of kinetic energy from the surface of *E. faecalis* cells when the phase lag occurs (cell adaptation phase). Cell kinetic energy is important for maintaining environmental changes when interacting with hosts and other bacteria²⁵.

Furthermore, the formation of biofilms by bacteria is related to the intensity of communication between pathogens in quorum sensing²⁶. *Citrus aurantifolia* may interfere with cell-cell communication by reducing cell surface conductivity, resulting in a decreased response to the environment. The impact of this process is the existence of bacterial cells independently, unable to express biofilm proteins perfectly in the early phase. Enterococcal surface protein (ESP) is reported as one of the *E. faecalis* cell surface proteins involved in biofilm formation²⁷.

Fig 3 and Fig 4 show the growth of *E. faecalis* colonies from both planktonic source isolates and the biofilm period primarily determined by the concentration of the *Citrus aurantifolia* extract. At concentrations 0.5, 2, and 6%, *Citrus aurantifolia* has bacteriostatic properties. Simultaneously, the highest concentration is bactericidal (12,14, and 16%) of both *E. faecalis*. Based on this phenomenon, it can be assumed that the bacteriostatic and bactericidal activity of the two *E. faecalis* the concentration and environmental changes influence faecalis isolates. Some of the environmental factors that

often affect the control of bacteria's growth phase are acidity (pH), temperature, macro and micronutrients, oxygen levels, and toxins²⁸. *Citrus aurantifolia* extract has a pH below 3, and this acidic pH provides a change in cell surface permeability that can interfere with membrane diffusion to adapt to the surrounding environment²⁹. Continuous interactions in an acidic atmosphere tend to cause lysis cells to die, or if they persist, they will increase their virulence³⁰. Under dead conditions, the acidic atmosphere may impair the cell membrane's diffusion, resulting in the intra-cell diffusion channel failing to function.³¹ This condition can stop the supply of oxygen and water absorption so that cells release cytoplasmic fluid as a start rather than cell death³².

Another reason is the possibility that *E. faecalis* bacteria can survive (bacteriostatic) from the influence of *Citrus aurantifolia* extract because *E. faecalis* can survive. After all, it has virulent proteins such as Secreted leaderless peptide signal (SIP) involved in quorum-sensing biofilms³³. The environment of this protein changes from acidic to alkaline conditions by inserting some protons through the membrane to maintain the cytoplasmic buffer capacity.³³ Besides, *E. faecalis* express virulent proteins such as lytic enzymes, cytolysin, and lipoteichoic acids to change the defense mechanism of host cells, which is beneficial to bacteria by inducing host cells through the autophagic mechanism³⁴. This ability to support bacterial stability efforts to defend themselves from antibacterial materials also suppresses the action of lymphocytes during adaptation to host tissue³⁵. So, on this basis, several *E. faecalis* cells are still alive even though they have been prepared with *Citrus aurantifolia* extract, especially in clinical *E. faecalis* isolates used in this study.

Citrus aurantifolia extract still provides an opportunity to form biofilms even though in limited frequency (Fig 5). The frequency of inhibition of *E. faecalis* biofilms in clinical isolates is in line with the log phase (growth) of *E. faecalis* regarding environmental changes (lag phase) to the influence of test material. Evans (2016) reported that some natural substances have different phyto-response properties. This property tends to be influenced by the responsiveness of the active compound³⁶. The signal channel of the active compound (oxygen and hydrogen bonding) reduces the communication channel signal between the bacterial cell surface protein and the surface matrix protein of the cell and host tissue³⁷. This effort causes bacterial cell fatigue, so it can be assumed that bacterial cells have difficulty expressing surface proteins involved in biofilm formation. Virulence activity of pathogenic cells such as *E. faecalis* correlates with cell death based on the findings of this study. In principle, every antibacterial compound has a complex

mechanism to prevent developing cells from suppressing growth³⁸.

Another finding from Fig 3 is that *Citrus aurantifolia* provides a tolerance limit (stationary phase) to the biological activity of *E. faecalis*, especially at a concentration of 12%. As commensal, this bacterial development must be controlled in the root canal to balance bacteria in the root canal to prevent infection. This test material can also lead to a death phase of *E. faecalis* for 18 hours (early phase). These results also indicate that during the death phase (bactericidal), the inhibition of biofilms is still stable (strong), meaning that biofilms that are spread (alive) after bacterial cells die can still be detected. It is possible the biofilm mass is not progressive enough to encourage the formation of quorum sensing communities by other bacteria. It is explained that the extract of *Citrus aurantifolia* and activating cell function in expressing biofilm-forming proteins can also prevent cell development by lysing *E. faecalis* cells in line with the data presented in Table 1.

Citrus aurantifolia extract can inhibit transglycosylation directly by binding to the transglycosylation domain of the enzyme (penicillin-binding protein)³⁹. This process is reported to prevent the formation of mass biofilms but not planktonic growth⁴⁰. This finding is consistent with the study results, where the inhibition of *E. faecalis* originated from planktonic was higher than that of the biofilm mass (Fig 3). This test material contains several acidic compounds that promote the acidic environment on the surface of bacterial cell walls in non-canonical D-amino acids. This acid interferes with transpeptidation and transglycosylation, inhibiting and spreading biofilms without affecting planktonic growth (free)⁴¹. According to the findings of this study, other specific studies must be conducted, including investigating the right concentration to avoid toxicity to host cells and determining which peptide domains are damaged due to failure of biofilm formation and *E. faecalis* cell development.

CONCLUSION:

Citrus aurantifolia has more potent biofilm inhibition against *E. faecalis* ATCC 29212 than *E. faecalis* clinical isolate (75%). The bacteriostatic and bactericidal properties of extracts of both *E. faecalis* from planktonic are more susceptible than those from biofilm mass sources.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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