



The growth and biofilm formation of *Enterococcus faecalis* in ethanol extract of *Citrus aurantiifolia* Indonesian species

[Crecimiento y formación de biopelículas de *Enterococcus faecalis* en extracto etanólico de especies de *Citrus aurantiifolia* de Indonesia]

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Abstract

Context: *Enterococcus faecalis* is reported as a biofilm bacterium involved in the pathogenesis of tooth root canal infection. The ability to grow and form biofilms is the reason these bacteria are difficult to be eliminated.

Aims: To analyze the antibacterial effect of *Citrus aurantiifolia* against the growth and biofilm formation of *E. faecalis*.

Methods: This study used *E. faecalis* ATCC 29212 and ethanol extract of lime peel (*Citrus aurantiifolia*). The *E. faecalis* growth was assessed by spectrophotometry, and biofilm formation was examined with 1% violet crystals also visualized by microscope (magnification 400×).

Results: *Citrus aurantiifolia* extract can inhibit the growth and biofilm formation of *E. faecalis* with varying quantities. Based on period incubations, a concentration of 6.25% had a better ability to suppress the growth of *E. faecalis* (<300 CFU/mL). The 75% concentration was the highest among other levels to inhibit the biofilms formation of *E. faecalis* that assumed the higher concentration of the extract, the most significant increase in inhibition of the biofilm formation of *E. faecalis*. Growth and biofilm formation of *E. faecalis* showed linearity in line with a concentration of 6.25% ($p < 0.05$; 0.000) with a relatively stable relationship ($r = 0.506$).

Conclusions: *Citrus aurantiifolia* extract can inhibit the growth of *E. faecalis* that is in line with the inhibition of the biofilm formation.

Keywords: bacterial growth; biofilm; *Citrus aurantiifolia*; *Enterococcus faecalis*.

Resumen

Contexto: *Enterococcus faecalis* se informa como una bacteria de biopelícula involucrada en la patogénesis de la infección del conducto radicular. La capacidad de crecer y formar biopelículas es la razón por la que estas bacterias son difíciles de eliminar.

Objetivos: Analizar el efecto antibacteriano de *Citrus aurantiifolia* frente al crecimiento y formación de biopelículas de *E. faecalis*.

Métodos: Este estudio utilizó *E. faecalis* ATCC 29212 y extracto etanólico de piel de lima (*Citrus aurantiifolia*). Se evaluó el crecimiento de *E. faecalis* mediante espectrofotometría y se examinó la formación de biopelículas con cristales violetas al 1% también visualizados al microscopio (aumento de 400×).

Resultados: El extracto de *Citrus aurantiifolia* puede inhibir el crecimiento y la formación de biopelículas de *E. faecalis* en cantidades variables. Basado en incubaciones de período, una concentración de 6,25% tuvo una mejor capacidad para suprimir el crecimiento de *E. faecalis* (<300 CFU/mL). La concentración del 75% fue la más alta entre otros niveles para inhibir la formación de biopelículas de *E. faecalis* que asumió la concentración más alta del extracto, el aumento más significativo en la inhibición de la formación de biopelículas de *E. faecalis*. El crecimiento y la formación de biopelículas de *E. faecalis* mostró linealidad en línea con una concentración de 6,25% ($p < 0,05$; 0,000) con una relación relativamente estable ($r = 0,506$).

Conclusiones: El extracto de *Citrus aurantiifolia* puede inhibir el crecimiento de *E. faecalis* que está en línea con la inhibición de la formación de biopelículas.

Palabras Clave: biopelícula; crecimiento bacteriano; *Citrus aurantiifolia*; *Enterococcus faecalis*.

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INTRODUCTION

Enterococcus faecalis is a Gram-positive commensal reported to be involved in the pathogenesis of tooth root canal infections. Besides, these bacteria play a role in endocarditis infections, bacteremia, urinary tract infections, and meningitis (Fiore et al., 2019). The impact of persistent *E. faecalis* infection can lead to canal failure treatment, which has implications for chronic or acute inflammation. It can cause tissue damage around the tip of the tooth root to lead to an abscess (Gomes and Herrera, 2018). Several virulence properties of *E. faecalis*, such as the ability to grow in an acidic and alkaline pH environment and form biofilms, have worsened the root canal infection (Jhajharia et al., 2015).

The *E. faecalis* is reported to grow in an acidic environment. This ability is influenced by the adaptation system on the cell surface. Gelatinase protein is an extracellular metalloprotease that acts to hydrolyze gelatin, collagen, and hemoglobin to maintain its survival. A number of these proteins of *E. faecalis* are reported as extracellular matrix involved in biofilm formation (Zheng et al., 2018). Based on this concept, it can be understood that the growth of *E. faecalis* has a close relationship with the intensity of biofilm formation as one of the strategies to survive and develop in the pathogenesis of dental root canal infection.

The *E. faecalis* is also called a biofilm bacterium due to its intensity of biofilm formation compared to other oral commensals such as *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* (Kuang et al., 2018). So that these bacteria are difficult to eliminate even though treatment has been done mechanically or chemically. Until now, the use of drugs still has limitations to eradicate the *E. faecalis* biofilm efficiently from intra-canal infections (Rosen et al., 2016).

Efforts to eliminate intra-canal biofilms, including using antimicrobial irrigation during root canal treatment, such as the use of sodium hypochlorite, but this accompaniment material has lim-

ited ability to remove biofilms from the root canals, can even cause persistent infections (Mohammadi et al., 2014). Moreover, ethylenediaminetetraacetic changes the structure and increase the microstrain of apatite crystals (Nasution et al., 2016). Lime (*Citrus aurantiifolia* (Christm.) Swingle, family *Rutaceae*) as one of the plants has been reported to have an anti-*E. faecalis* effect because it can reduce tolerance to the influence of critical acids (pH<3) (Mubarak and Soraya, 2018). Therefore, efforts should be made to suppress the growth and formation of *E. faecalis* biofilm to achieve adequate disinfection of the root canal system.

Unbalanced growth as a commensal and an increase in *E. faecalis* biofilm can pose a severe dental health threat because, in this phase, *E. faecalis* is difficult to prevent by antibacterial agents and the immune system (Belkaid and Hand, 2014). Penetration failure can be attributed to various factors, including acceleration of growth and increased maturation of the biofilm matrix, which causes an increase in multi-bacterial resistance to biofilm formation in the root canal of the tooth (Khalifa et al., 2016). *Citrus aurantiifolia* extract is expected to be able to suppress the growth and biofilm formation of *E. faecalis*. It may be possible to be used as a natural material for the treatment of root canal infection. This study evaluates the potential of *Citrus aurantiifolia* as an antibacterial agent for the growth and formation of *E. faecalis* biofilms in the root canals of teeth *in vitro* based on the spectrophotometric analysis.

MATERIAL AND METHODS

Laboratory experiment research (*in vitro*) was conducted in 2019 at the Research Laboratory of the Faculty of Veterinary, Syiah Kuala University, Banda Aceh, Indonesia. The *E. faecalis* ATCC 29212 and ethanol extract of *Citrus aurantiifolia* were used as test material, and 0.2% chlorhexidine (CHX) (Minosep, Medikahealthcare, Depok, Indonesia) was utilized as a positive control. Growth inhibition and biofilm formation were examined using spectrophotometry based on a specific wavelength.

Plant material

Lime (*Citrus aurantiifolia*) was obtained from PT Hilya Agri Jaya Lime Gardens, Aceh Jaya District, Aceh, Indonesia 23653. GPS Coordinates 4°31'13.7"N 95°52'21.9"E, in 2019, and identified by one the authors (Basri A. Gani). The voucher specimen (JN-EE-04) was deposited at the Oral Biology Laboratory, Faculty of Dentistry, Syiah Kuala University, Aceh, Indonesia.

Extract preparation

The *Citrus aurantiifolia* was extracted using 96% ethanol (Mubarak et al., 2018). One kg of peel was washed thoroughly using distilled water, cut into small pieces, and airdried for 48 h. The extraction was performed by the maceration method. A total of 500 g of lime peel was placed in a dark bottle and was soaked with 1 L of ethanol for 6 hours while shaking and then leaving it for the next 18 h. Furthermore, the lime peel extract was filtered with sterile filter paper. The extract was then evaporated using a rotary vacuum evaporator (Buchi, Switzerland). The temperature was set at 50°C with a pressure of 20 Psi (pounds of force per square inch) and a speed of 120× gravity (g) for 12 h. The concentrated extract was then prepared to be the following concentrations 6.25, 12.5, 25, 50, and 75% (w/v).

Enterococcus faecalis growth

Enterococcus faecalis was cultured on brain heart infusion (BHI) media (Merck KGaA, Darmstadt, Germany) for 48 h, then equalized with McFarland 0.5 (1.5×10^8 /mL). *E. faecalis* growth was measured using a spectrophotometer (Chang et al., 2017). In a 96-well plate was deposited a 50 µL critical saliva (triple serial), incubated for 15 min and washed with 50 µL by PBS (phosphate-buffered saline) at pH 7 (Merck KGaA, Darmstadt, Germany). A volume of 25 µL *E. faecalis* was added and incubated for 15 min. Furthermore, 100 µL of *Citrus aurantiifolia* extract was added, shaken at 200× g for 5 min, and incubated for 24, 48, and 72 h at 37°C. Growth measurements of *E. faecalis* were analyzed based on turbidity, which began by removing all test material mixtures from *E. faecalis*

after incubation. Each well was filled with 125 µL PBS with a pH of 7 and then shaken for 15 min at 500×g. The turbidity of the solution was measured by ELISA reader spectrophotometry (Bio-Rad, USA) at a wavelength of 620 nm as a reference to the growing quantity of *E. faecalis*. Determination of the calibration of the optical density (OD) values on the number of *E. faecalis* colonies was adopted by Sutton (2011).

Biofilm formation assay

The *E. faecalis* biofilms formation assessment was carried out by the 1% crystal violet method on 96-well plates, based on Gani et al. (2017). The preparation of the *Citrus aurantiifolia* extraction test material was initiated by making concentrations of 6.25, 12.5, 25, 50, and 75%. A 96-well plate was coated with 100 µL TSB medium, then incubated for 15 min, then washed with PBS (pH 7.0) and poured into each well 25 µL of *E. faecalis* and then adapted at room temperature for 15 min. Furthermore, saliva and histatin-5 test materials were added to the different tests with triple serial. Then Homogenized between assay material with *E. faecalis* on the shaker, at 500×g for 10 min and incubated for 24, 48, and 72 h. The assessment of the biofilm formations of *S. mutans* began by removing all the solutions in wells and then washing them with PBS and dishwasher at 500 rpm for 10 min. This treatment was repeated twice. Then, 150 µL of 1% violet crystal was poured into each well. The crystal violet dye was homogenized with protein biofilm on a shaker at 200 rpm for 10 min. Subsequently, each well was washed with 150 µL PBS for 5 min. Then it was discarded and continued washing with 150 µL of 70% ethanol for 1 min. After that, 96-well plates containing biofilms were marked by the absorption of violet crystalline dyes and incubated at room temperature for 15 min. The quantity of biofilm was measured by spectrophotometry (560 nm).

Visualization of biofilm mass of E. faecalis

The glass surface was made rough with a 2000 grid. Then, it was soaked in saline for 60 min. Afterward, the glass was dried above the shaker at 500×g. The surface was blocked 1 × 1 cm and sub-

sequently covered by 200 mL of *Citrus aurantiifolia* extracts (6.25, 12.5, 25, 50, and 75%, and CHX 0.2%). The glass was incubated for 15 min, followed by adding *E. faecalis* 20 µL (1:10) and reincubated for 12, 24, and 48 h. Visualization of the biofilm mass was modulated by removing the test material with bacteria that was washed with buffer saline and allowed to stand at room temperature for 15 min. Then crystal violet 1% 200 µL was administered for 5 min and then was washed with buffer saline. It was incubated furthered at 37°C for 20 min, and the glass was then observed under the Optilab Viewer assisted microscope (Miconos, Optilab Advance, PT Miconos, Yogyakarta, Indonesia), magnification 400×.

Statistical analysis

All experiments were carried out in technical and biological triplicates. One-way ANOVA and Kruskal-Wallis analyzed the data growth and the biofilms formation of *E. faecalis* in various concentrations in the ethanol extract of *Citrus aurantiifolia*. Paired t-test to the analysis of both with $p < 0.05$ was considered statistically significant.

RESULTS

Fig. 1 shows that in general, all concentrations of *Citrus aurantiifolia* extract can inhibit the growth of *E. faecalis* with varying quantities. Based on the incubation period of this test material, it is more stable to suppress the growth of *E. faecalis* at an incubation period of 12 h for the concentrations of 6.25, 12.5, 25, 50, and 75%, which range from 300 CFU/mL (Table 1). In Fig. 2, it is exhibited that, in all concentrations of *Citrus aurantiifolia*, there is an inhibitory formation of *E. faecalis* biofilms. At 6.25%, it still shows an inhibition, especially during the 12 h incubation period. While the highest concentration, highest inhibition obtained at 24 and 48 h of incubation periods. Fig. 3 clarifies the profile of biofilm after interacting with ethanol extract of *Citrus aurantiifolia*. The higher the concentration of the extract, the higher the inhibition of the biofilm formation of *E. faecalis*. Fig. 4 shows that the ability to inhibit biofilm formation has linearity with the *E. faecalis* growth in *Citrus aurantiifolia* extracts. The graph shows the incubation periods in 6.25% had better consistency based on graph linearity.

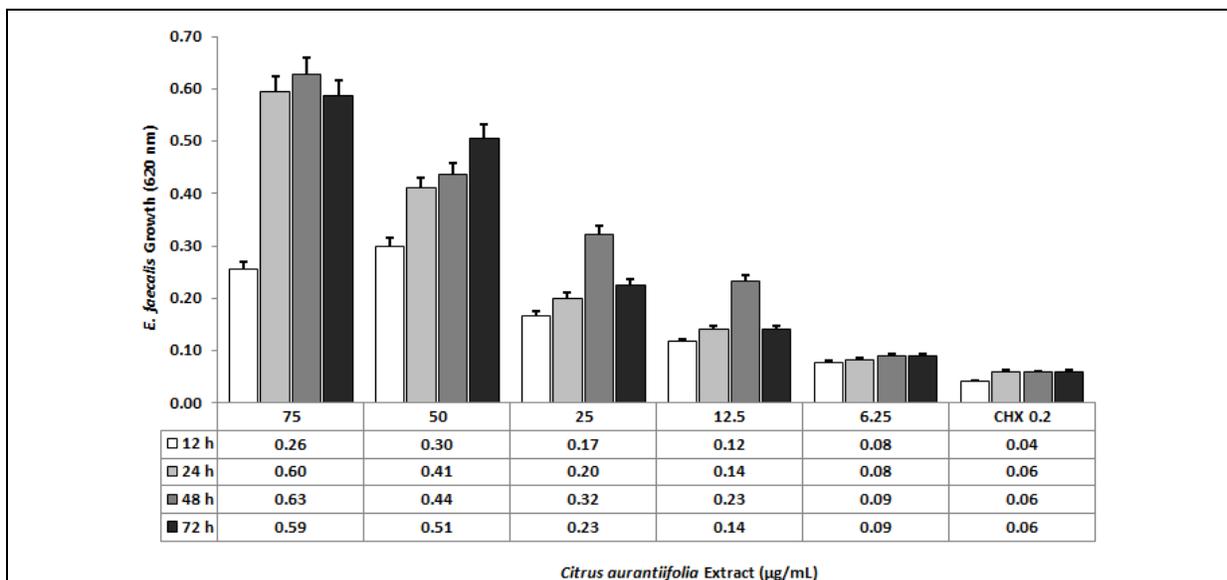


Figure 1. The growth of *E. faecalis* in *Citrus aurantiifolia* extract was calibrated by spectrophotometry (620 nm).

The concentration of 6.25% has a better ability to suppress the growth of *E. faecalis* (<300 CFU; 0.5 McFarland) than other concentrations. Whereas the incubation period of 12 h is the best time, the role of *Citrus aurantiifolia* extract suppresses the growth of *E. faecalis* at all concentrations. Data represent means ± SD, (n=3). Nevertheless, One-way ANOVA showed that there was no significant difference in the growth of *E. faecalis* ($p > 0.05$: 0.671) between the incubation periods of 12, 24, 48, and 72 h. Whereas, based on the Kruskal-Wallis of concentrations analysis shown the significant difference ($p = 0.001$) with a strong relationship ($r = 0.955$). CHX: Chlorhexidine.

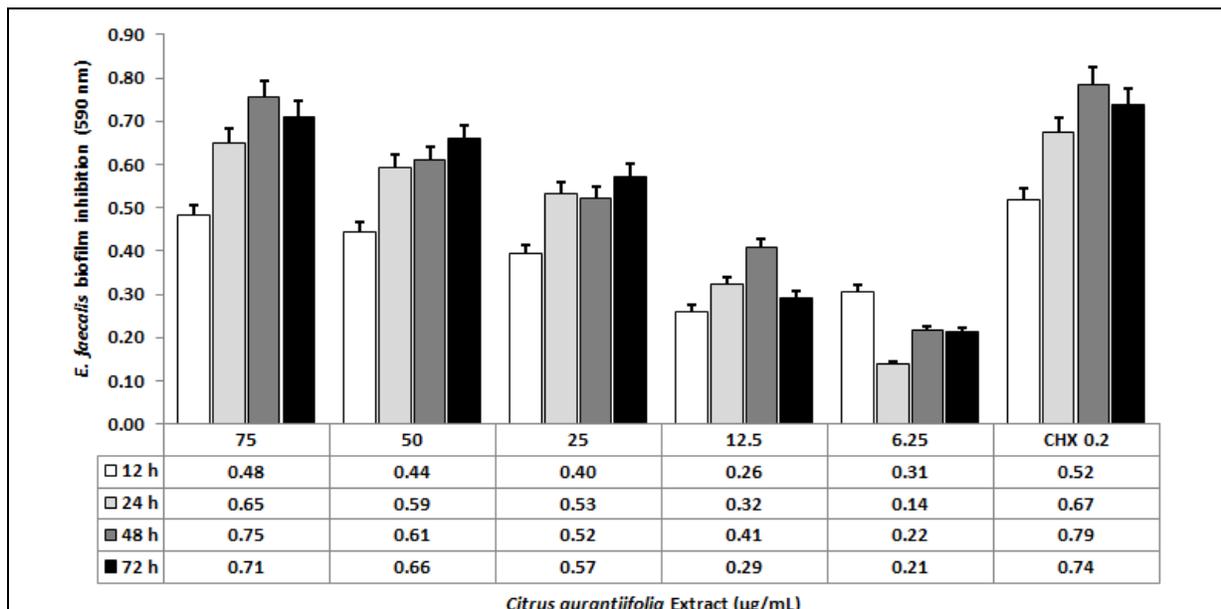


Figure 2. Inhibition biofilm of *E. faecalis* by *Citrus aurantiifolia* extract based on time and temperature. The concentrations of *Citrus aurantiifolia* were able to inhibit the biofilm formation of *E. faecalis*. The 75% concentration is the best among other concentrations to inhibits the formation of biofilms of *E. faecalis*. Data represent means ± SD, (n=3). The One-way ANOVA analysis showed no significant difference (p=0.50) between the incubation periods of 12, 24, 48, and 72 h, while based on the concentration, there is a significant difference (p=0.000) with a weak relationship (r=0.285). CHX: Chlorhexidine.

Table 1. The *E. faecalis* growth in *Citrus aurantiifolia* extract with spectrophotometric calibration.

Treatment (%)	<i>E. faecalis</i> growth 12 h			<i>E. faecalis</i> growth 24 h			<i>E. faecalis</i> growth 48 h			<i>E. faecalis</i> growth 72 h		
	OD	CFU	MF Scale									
CAE												
75%	0.26	600	2	0.60	1800	6	0.63	1800	6	0.59	1500	5
50%	0.30	900	3	0.41	1200	4	0.44	1200	4	0.51	1500	5
25%	0.17	300	1	0.20	600	2	0.32	900	3	0.23	600	2
12.5%	0.12	300	1	0.14	300	1	0.23	600	2	0.14	300	1
6.25%	0.08	<300	0.5	0.08	<300	0.5	0.09	<300	0.5	0.09	<300	0.5
CHX 0.2%	0.04	<300	0.5	0.06	<300	0.5	0.06	<300	0.5	0.06	<300	0.5

CAE: *Citrus aurantiifolia* extract; MF: McFarland; OD: Optical Density; CFU: Colony-forming unit; CHX: Chlorhexidine.

Based on the period incubation, the *E. faecalis* growth increased in high concentrations of *Citrus aurantiifolia* (25, 50, and 75%), with an exponential phase at 48 h. Whereas, on the 12.5 and 6.25% concentrations decreased the growth of *E. faecalis* based on calibrated from the CHX 0.2% solution as standard gold in root canal treatment (≤300 CFU/mL). Statistically, all concentrations showed different levels of *E. faecalis* growth at each of interval incubation times but did not show a significant difference (p=0.671).

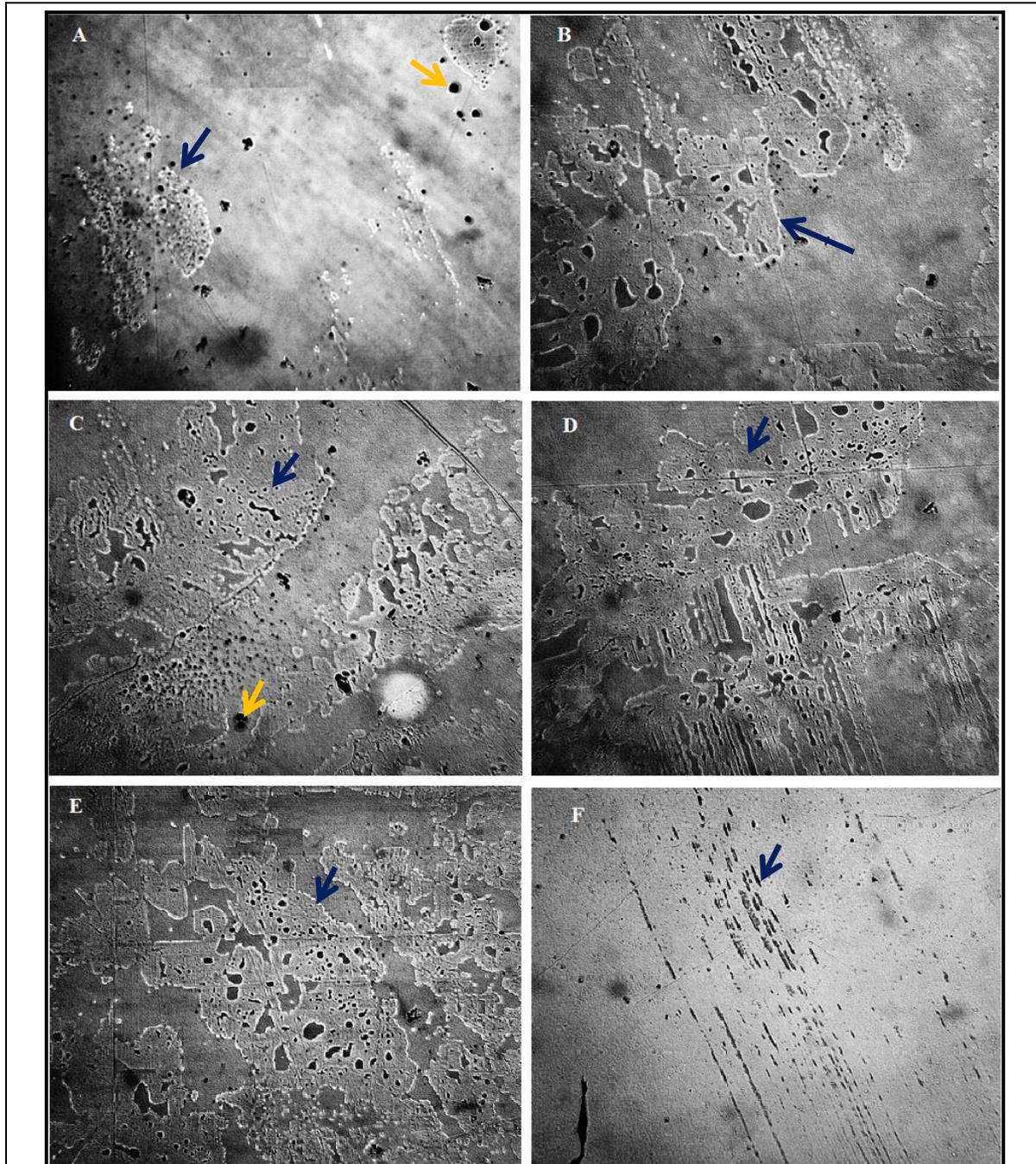


Figure 3. The biofilm formation of *E. faecalis* on the glass for 24 h after being interacted with *Citrus aurantiifolia* concentrations (A) 75%, (B) 50%, (C) 25%, (D) 12.5%, (E) 6.25%, and chlorhexidine (F) 0.2%.

Blue arrow (Biofilm mass), a yellow arrow (*E. faecalis* cells generated through lyses). Generally, the biofilm mass of *E. faecalis* degrades as concentrations of *Citrus aurantiifolia* increase (magnification 400×). The changes in the inhibition of the formation of biofilms for all concentrations and incubation periods indicate these two factors determine the strength of biofilms expressed by *E. faecalis*.

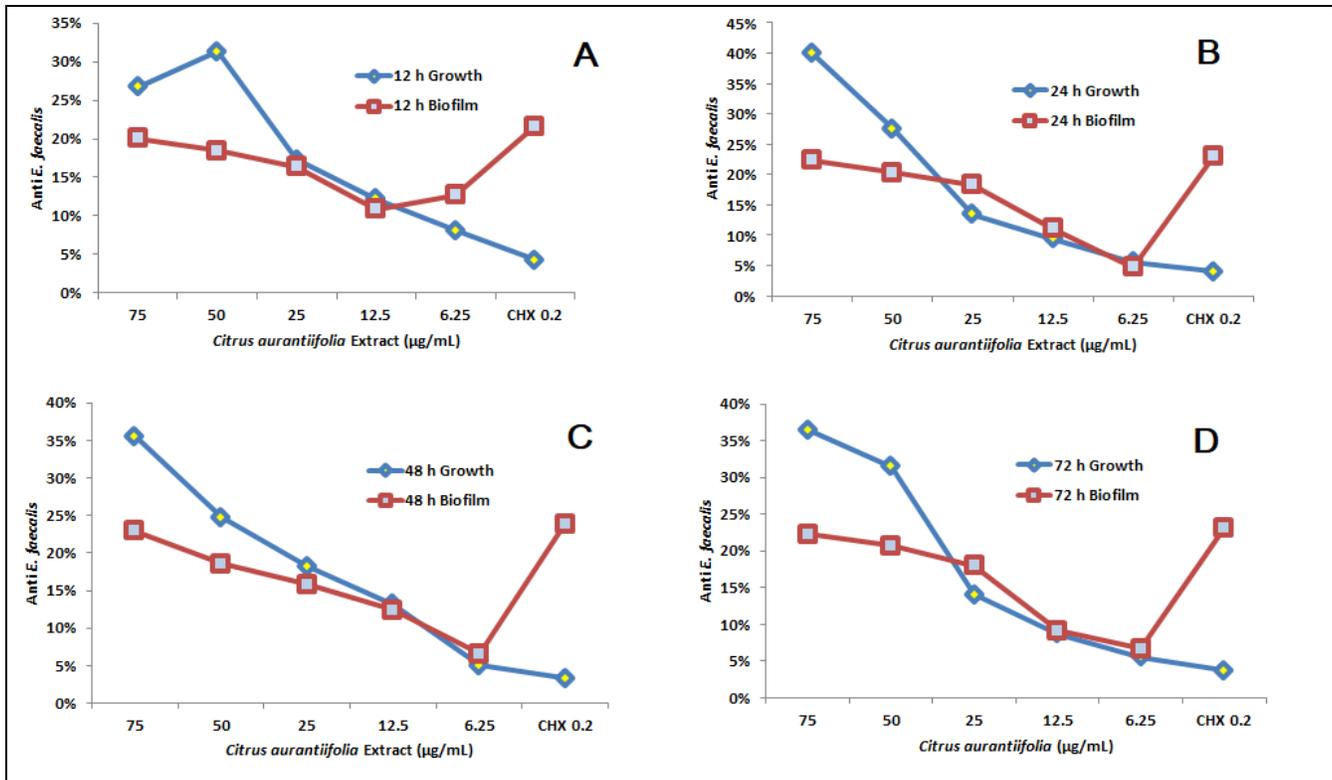


Figure 4. The linearity of *E. faecalis* growth with the ability to biofilm formation of *E. faecalis* affected by *Citrus aurantiifolia* extracts during 12 h (A), 24 h (B), 48 h (C), and 72 h (D).

Based on the concentration and incubation, shown that the growth of *E. faecalis* is in line with the ability of the concentration of the *Citrus aurantiifolia* extract to inhibit the biofilms formation. The higher the growth of *E. faecalis*, the lower the inhibition of biofilms and vice versa. The ability to suppress the growth of *E. faecalis* was in line with the ability to inhibit the formation of biofilms, although in general other higher concentrations inhibited the formation of biofilms but not consistent because it has a low ability to suppress the growth of *E. faecalis*.

Data represent means ± SD, (n=3). Paired T-test statistically showed growth of *E. faecalis* in line with its ability to inhibit the formation of biofilms that were significantly different (p=0.000) with a relatively strong relationship (r=0.506).

DISCUSSION

This study evaluates an ethanolic extract of the *Citrus aurantiifolia* peel as an antibacterial agent, explicitly assessing the biological effects in inhibiting the growth and formation of *E. faecalis* biofilms so that they can benefit as candidates for root canal irrigation agents. The high growth and biofilms formation of *E. faecalis* cause these bacteria to be persistent with the accompanying material, so these bacteria are complicated to be eliminated.

Fig. 1 shows that the *Citrus aurantiifolia* extract at various concentrations can inhibit the growth of *E. faecalis* according to the concentrations and incubation period. The results of the study indicate that the lowest concentration still demonstrated

optimal performance in inhibiting the growth of *E. faecalis*. The extract at the following concentrations of 6.25, 12.5, 25, 50, and 75% with the number of colonies based on spectrophotometric examination ranging ≤300 CFU/mL (Table 1). It is a strong indication that the bacteria interacted with *Citrus aurantiifolia* have not developed because the standard colonies used when testing use the McFarlan Standard 0.5 with a colony range <300 CFU/mL. Thus it can be hypothesized that *Citrus aurantiifolia* is bacteriostatic (inhibits growth).

Based on the tolerance concept, *Citrus aurantiifolia* has shown significant tolerance properties to control the balance of *E. faecalis* bacteria, which are commensal in the root canals of the tooth (Mubarak and Soraya, 2018). Therefore, it can be assumed that *Citrus aurantiifolia* is a

potential candidate for accompanying material. The root canal treatment is one of the main goals of preventing the development of bacteria from the root canal system to help to treat or preventing root canal infections (Samiei et al., 2016). Several *Citrus aurantiifolia* concentrations have a very beneficial effect in inhibiting the growth of *E. faecalis*, which is related to the ability of several active components of *Citrus* species to damage cell membranes (Oliveira et al., 2014).

The concentration of extracts has a strong influence on the penetration power of the cell membrane. It has related to the number and size of molecules when penetrating bacterial cell walls (Martínez-Sanz et al., 2015). Therefore, it is possible that at the lowest concentration, *Citrus aurantiifolia* extract may cause cell membranes to leak into the cytoplasmic components, thereby causing cell death (Chamberlin et al., 2019). It is challenging to solve because the surface membrane of Gram-positive bacteria is not entirely waterproof. The *E. faecalis* is a Gram-positive bacterium with porin proteins in the membrane layer. These proteins created the large channels enough to allow passage of molecular mass below 600 kDa. Several active ingredients, such as the phenolic compounds contained in *Citrus aurantiifolia*, which is substituted, provides penetration into the periplasmic space and cytoplasmic membrane (Lepore et al., 2011). This arrangement can facilitate the antibacterial ion entry into the cell (Rajagopal and Walker, 2015). Another possible reason for this is that several active compounds of *Citrus aurantiifolia* can inhibit growth, which is influenced by the presence of negative charge. The negatively charged molecules of this active plant compound have a higher affinity for the release of positive ions in the bacterial cell wall, thereby causing accumulation and increase in ion absorption, which then causes intracellular damage and interferes with bacterial growth (Eckhardt et al., 2013).

As shown in Fig. 2, all concentrations of *Citrus aurantiifolia* inhibited the formation of *E. faecalis* biofilms. The concentration of 6.25% was the minimum concentration but still affected the inhibition, especially at the 12 h of the incubation

period. The inhibition of biofilm corresponds to the incubation period. It can be hypothesized that *Citrus aurantiifolia* is acidic (pH<3) (Mubarak et al., 2018), and it is possible to influence and prevent the formation of protein matrix biofilms. This ability is empowered by several active compounds contained in *Citrus aurantiifolia* such as caryophyllene, beta-caryophyllene, and bicyclogermacrene, which act as antibacterial. Caryophyllene is known to have strong selective cytotoxic properties, and bicyclogermacrene can inhibit the growth of gram-positive bacteria by damaging the structure of cell walls (Selestino Neta et al., 2017). It is in line with the results of this study, where the anti-biofilm activity was increased at incubation periods of 48 and 72 h, meaning that several active compounds from *Citrus aurantiifolia* can suppress the maturation of biofilm matrices to spread in the area of colonization of other biofilm bacteria. Bacteria biofilm is ten times up to 1000 times more resistant to the antimicrobial than planktonic bacteria (free-living) (Balcázar et al., 2015). These potentials can be assumed that *Citrus aurantiifolia* can prevent the colonization of the bacteria involved in the pathogenesis of dental root canal infections.

In the root canal environment, *E. faecalis* bacteria play an essential role in forming biofilm (Pourhajbagher et al., 2016). Therefore, *E. faecalis* biofilm is considered an appropriate model for testing new antimicrobial treatments on the results of the study obtained changes in the inhibitory formation of *E. faecalis* biofilms in all *Citrus aurantiifolia* concentration and variations in the incubation period. These two variables of analysis determine the strength of biofilms expressed by *E. faecalis* after being influenced by *Citrus aurantiifolia*. According to Roy (2018), several D-amino acid compounds in natural materials such as *Citrus aurantiifolia* have been reported to inhibit the spread of biofilms without affecting the growth of new planktonic bacteria (Roy et al., 2018). But so far, our studies on the effectiveness of endodontic biofilms have never been evaluated. Besides, the hydrophobicity of *Citrus aurantiifolia* contributes to the breakdown of lipids in cell membranes and disrupts the permeability of cell walls. Moreover,

active compounds such as essential metabolites (folic acid) can prevent the enzymatic reactions that interfere with synthesis (Chouhan et al., 2017).

Fig. 3 shows the biofilm mass of *E. faecalis* declined in 24 h of incubation. It is assumed that the *Citrus aurantiifolia* not only blocks the early phase of biofilm formation but also capable of preventing the maturation of the biofilm matrix and delaying the spread of quorum sensing. One of the reasons is the *Citrus aurantiifolia*, possibly disturbing the active transport system of bacteria cell membranes. It has an impact on the increase of the surface pressure of bacterial membranes to interfere with membrane surface proteins when interacting with the environment (Nazzaro et al., 2013).

In Fig. 4 illustrates that the growth inhibition is in line with the inhibition of the biofilms formation of *E. faecalis* in *Citrus aurantiifolia* extracts, especially at a concentration of 6.25%. Whereas at other concentrations, more inhibitory biofilm formation is higher, but not consistent, because it has a low ability to suppress the growth of *E. faecalis*. This phenomenon can be interpreted that the lowest concentration of *Citrus aurantiifolia* has the appropriate number and size of molecules to change the cell membrane active transport system. This activity's impact can facilitate the active compound binding to biofilm proteins found on the surface of the cell wall (Koo et al., 2017). However, this study's results were found at the highest concentrations (50 and 75%). Inhibitory growth is not in line with the inhibition of the formation of biofilms of *E. faecalis*. There are reports of similar results to those obtained in this study. As reported by Kooltheat et al. (2016), Kaffir lime leaves extract inhibited *S. mutans* in line with the inhibitory power of biofilm formation, which was confirmed by biofilm genes.

Many research results suggest that *E. faecalis* bacteria have a mosaic series of anionic surface domains that facilitate the binding of several active antibacterial compounds. Stronger interactions between ligands and receptors can increase relatively high cell toxicity. Besides, several reactive oxygen species (ROS) such as hydroxyl

radicals that are negatively charged easily penetrate cell membranes (Russell and Cotter, 2015). The antibacterial role in inhibiting the growth and biofilms formation using peptidoglycan, activating autolysis-genes on the cell membranes, and changing cell surface proteins' structure. Other strategies to increase the negative charge and oxygen solubility through ROS mechanisms. These mechanisms mediated by lipid peroxidation and reactive electrophile species (Büttner et al., 2015).

CONCLUSIONS

The ethanol extract of *Citrus aurantiifolia* has the ability to inhibit the growth and biofilm formation of *E. faecalis*. The capability to inhibit growth and biofilm formation shows linearity in its action based on the incubation periods. Therefore it is necessary for further study, the biostatic properties of *Citrus aurantiifolia* in the root canal of the teeth associated with protection against *E. faecalis* infection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Soraya C	Mubarak Z	Gani BA
Concepts or ideas	x		x
Design	x		x
Definition of intellectual content		x	
Literature search		x	
Experimental studies	x		x
Data acquisition	x		
Data analysis	x		x
Statistical analysis			x
Manuscript preparation	x		x
Manuscript editing	x		x
Manuscript review	x	x	x

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