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RESEARCH ARTICLE

In vitro antibiofilm evaluation of *ocimum basilicum* extracts against *enterococcus faecalis*

Vera Yulina*✉, Yetty Herdiyati, Eka Chemiawan****

*Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Syiah Kuala, Banda Aceh, Indonesia
**Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Padjadjaran, Bandung, West Java, Indonesia
*Jl Hamzah Fansuri Kopelma Darussalam, Banda Aceh, Aceh, Indonesia; ✉ correspondence: vera.yulina@usk.ac.id

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ABSTRACT

Enterococcus faecalis is the main pathogenic bacterium in the root canal which is resistant to various antibacterial agents. Recently, the discovery of a new antibacterial agent research focus in herbal dentistry, one of which is basil leaves. Basil (kemangi) leaves (*Ocimum basilicum*) are herbs that have various active components as anti-biofilm agents. This study aims to evaluate the effectiveness of antibacterial potential of *O. basilicum* extract against the biofilm formation by *E. faecalis*. This research uses true experimental laboratory design. The kemangi leaves were gradually extracted with various polarity based organic solvents (*n*-hexane and ethyl acetate). The evaluation of the anti-biofilm activity of the extracts against *E. faecalis* aimed to determine the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) values. Calcium hydroxide paste (calcipex) was used as a positive control. The assay data were analyzed using an independent paired T-test. Ethyl acetate, *n*-hexane extract of *O. basilicum*, and calcipex inhibited biofilm formation by *E. faecalis* with MBIC values of 100%, 100%, and 75.20% at the concentrations of 2.5%, 10%, and 3.13%, respectively. The MBEC values were 100%, 100%, and 71.01% at the concentrations of 5%, 2.5%, and 3.13%, respectively. The statistical analysis by ANOVA test on MBIC and MBEC showed significant differences with p-values < 0.05. Further statistical valuation with post hoc analysis showed highly significant differences with p-values < 0.01. This study has found that *O. basilicum* leaves extracts have the capacity and ability to inhibit and eradicate *E. faecalis* biofilms.

Keywords: *O. basilicum;* biofilm; *Enterococcus faecalis*; MBIC; MBEC

INTRODUCTION

Root canal treatment in primary teeth is performed to maintain deciduous teeth until their permanent replacement teeth erupt.[1,2] The success of root canal treatment, especially in primary teeth which have complex root canal anatomical characteristics, requires more than just instrumentation to be successful.[3] In root canal treatment procedures, chemical-mechanical preparation only functions for debridement, whereas bacterial colonization can be formed again both in the pulp chamber, root canal, and periapical tissue. This bacterial colonization within the root canal tends to form a community known as a root canal biofilm.[4–6]

Root canal biofilm is a complex structure consisting of a collection of microorganism cells, specifically the bacterium *Enterococcus faecalis* which is attached to the root canal wall and is covered by an exopolysaccharide matrix released by these bacteria.[7] Root canal biofilms are often found at the root canal of teeth with primary infection and apical periodontitis after root canal treatment. The anatomical and geometric complexity of the root canal such as the presence of an isthmus can protect biofilms that attach to the root canal wall from debridement procedures.[8]

Enterococcus faecalis is a major pathogenic bacterium in root canal biofilms that can survive in extreme environments such as high alkaline conditions.[9] Virulence of this bacterium can attach, invade dentinal tubules, and form communities in biofilms that are difficult to destroy from the root canal system and periapical tissue through instrumentation alone.[4,10] An effective

medicinal material is required against the main pathogenic bacteria of these root canal biofilms.[1] The medicinal ingredient that is often used as a gold standard in root canal treatment is calcium hydroxide, but calcium hydroxide can increase the adhesion of *E. faecalis* to collagen (the main organic component of dentin) which increases the rate of tubular invasion and thus making this bacterium resistant to further disinfection.[11,12] The increase in bacterial resistance to synthetic medications has led scientists to examine various herbal ingredients that have antibacterial effects,[13] low toxicity, high biocompatibility, and easily obtained; one of which is kemangi or basil (*Ocimum basillicum*) leaves.[14–16]

Phytochemical screening of the *O. basilicum* leaf confirmed the presence of phenolic, flavonoids, triterpenoids, saponins, and tannins.[14] The essential oil component of this plant was identified as camphor, limonene, thymol, citral, α-linalool, β-linalool, and estragole.[17] It has been reported in another study that it has several compounds including linalool, estragole, methyl eugenol, and 1.8-cineole.[18] This antibacterial mechanism occurs by binding phenol compounds with bacterial cells, which will interfere with membrane permeability and transport processes. This results in the loss of cations and macromolecules from cells, causing disruption or death of cell growth.[19] The hydrophobic compound of eugenol was identified as a main antibacterial agent of basil leaves with the mechanism that occurs by separating fatty tissue and mitochondria in the bacterial cell membrane and changing the cell structure. This enables eugenol to enter the bacterial cell membrane.[20] Based on the data of various active compounds in this plant with an antibacterial effect against pathogenic oral bacteria, this study aims to evaluate of effectiveness of antibacterial property of basil leaves extract against the formation of *E. faecalis* ATCC 29212 biofilm by in vitro assay. The data may serve as a scientific basis for considering this plant as a potential alternative of natural ingredients for root canal medicaments in the treatment of primary tooth pulp.

MATERIALS AND METHODS

Fresh leaves of kemangi (*O. basilicum* L.) were collected in January 2018 from local farmers in Ciwidey, Bandung Regency, West Java Province, Indonesia. The voucher specimen was determined and deposited at the Plant Taxonomy Laboratory, Department of Biology, Universitas Padjadjaran, Bandung, Indonesia.

The distilled organic solvents of methanol, ethyl acetate, and *n*-hexane were used for sample extraction, while for samples assay and chemical analysis, pro-analytical grade chemicals purchased from Merck and Sigma–Aldrich were used. The instruments in this study included a laminar air flow, an incubator (Memmert, IN55), an anaerobic jar (Oxoid, AG0025A), an autoclave, a microplate reader (Biochrom EZ read 400, 80-4001-40), a micropipette (Eppendorf, 3120000062 and 3120000054), and a microplate reader.

The extracts of basil leaves were prepared by repeated maceration methods using distilled methanol for 3 x 24 hours. The extract solutions were then filtered and evaporated using a rotary vacuum evaporator R-100 (Buchi-Germany) at 40 °C to obtain concentrated methanol extract. Following this, the crude methanol extract was fractionated by dissolving it into distilled water and partitioned using *n*-hexane solvent to obtain *n*-hexane fraction and water fraction. The water fraction was re-partitioned using ethyl acetate to yield ethyl acetate and water fraction. The fraction of *n*-hexane and ethyl acetate was evaporated by solvents using a rotary vacuum evaporator, then each fraction was made into concentrations of 1.25, 2.5, 5, 10, and 20%.

Enterococcus faecalis ATCC 29212 was used for antibacterial and anti-biofilm testing with Muller Hinton broth and Muller Hinton agar as mediums, chlorhexidine (purchased from Merck Co. Ltd. and Sigma-Aldrich, Onemed hexidine – East Java, Indonesia) as a positive control, and anaerobic jar antibacterial assay. The research received approval and recommendations from the Health Research Ethics Committee, Faculty of Medicine, Padjadjaran University (letter number 10/UN6.KEP/EC/2020; issued in Bandung on January 2, 2020).

The rejuvenation assay preparation was conducted by increasing the bacteria in the BHIB medium, then incubated at 37 °C for 24 hours in an anaerobic atmosphere using an anaerobic jar. The turbidity was adjusted to 0.5 McFarland standard (0.5 x 108 CFU / ml), and 100 µL *E. faecalis* ATCC 29212 was suspended on 100 µL of artificial saliva media (Biochemistry Laboratory, FK Unpad). BHIB was then added in 96 well microplates. Subsequently, it was incubated at 37 °C for 48 hours in an anaerobic atmosphere using an anaerobic jar. The negative control (without treatment) and positive control in the form of calcium hydroxide paste (calcipex II) were made into concentrations of 3.13, 6.25, 12.5, 25, and 50%.[21]

Minimum biofilm inhibitory concentration assay (MBIC) was carried out by inserting the test solution and culture of *E. faecalis* biofilm in a microtube. It was then incubated for 48 hours at 37°C, and the remaining saliva and bacterial culture were discarded and washed twice with phosphate buffer saline liquid (PBS). The safranin solution of 50 µL at 0.1% (w/v) was added to each well as a coloring agent and incubated for 15 minutes. The dye was then removed, and each well was washed with sterile water to remove excess safranin. Finally, 150 µL ethanol was added to each well and incubated for 30 minutes to suspend biofilm. Measurements were made with a microplate reader at a wavelength of 490 nm. Testing was replicated three times. The minimum inhibitory concentration of biofilm is defined as the lowest concentration of the sample solution that can inhibit biofilm growth.[22]

The minimum biofilm eradication concentration test (MBEC) was conducted by making liquid media with the addition of artificial saliva with a final concentration of 0.5%, then the turbidity of the bacteria was calculated, and the media was used. Into 96 well microplates, 200 mL of *E. faecalis* culture was added with 0.5 McFarland turbidity. After incubation for 48 hours, the bacterial culture was removed. Then each well was washed

Table 1. Anti-biofilm activity of Ocimum basilicum extracts against E. faecalis biofilm formation.

Groups	Concentration (mg/ml)	Absorbance			Average	Inhibition (%)
		OD I	OD II	OD III		
Ethyl Acetate extract	20	-0.343	-0.322	-0.177	-0.281	100
	10	-0.696	-0.694	-0.697	-0.696	100
	5	-0.295	-0.353	-0.177	-0.275	100
	2.5	-0.089	-0.092	-0.086	-0.089	100
	1.25	0.047	0.003	0.016	0.022	-
n-Hexane extract	20	O/R	-0.811	-0.156	-0.484	100
	10	O/R	-1.018	-0.171	-0.595	100
	5	0.577	0.639	0.203	0.473	-
	2.5	0.889	0.809	1.337	1.012	-
	1.25	0.591	0.454	0.947	0.664	-
Calcipex	50	-0.009	-0.010	-0.021	-0.013	100
	25	-0.029	-0.014	-0.005	-0.016	100
	12.5	-0.004	-0.077	-0.106	-0.062	100
	6.25	-0.021	-0.005	-0.013	-0.013	100
	3.13	0.004	0.012	0.002	0.006	75.21
Negative Control	Without treatment	0.016	0.016	0.016	0.016	-

OD: Optical Density (Turbidity absorbance value); O/R: cannot be read

with 300 µL of PBS, treated with 200 ml of the test solution, and incubated for 30 minutes. Calcipex II was used as a positive control and a negative control without treatment. After incubation, each well was washed with 300 µL of PBL of. One hundred and fifty µL of safranin was added to each well as a coloring agent and incubated for 15 minutes. The dye was then removed, and each well was washed with 300 µL of water for 3 times. Finally, 150 µL of ethanol was added to each well, incubated for 30 minutes, and measured using a microplate reader at a wavelength of 490 nm. The minimum eradication concentration of biofilm is determined by identifying the lowest concentration of sample solution that can destroy biofilms.[22] Data were analyzed using Microsoft Excel software using analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test at a significance level of $p < 0.05$.

RESULTS

The leaves of kemangi (13.6 kg) were extracted with MeOH for 3 x 24, filtered, and evaporated in a vacuum to give a concentrated extract of 103.2 g. Then, the extract was repeatedly partitioned between *n*-hexane-H_2O and EtOAc-H_2O, resulting in *n*-hexane and EtOAc extracts of 21.94g and 13.76 g, respectively.

Tests were carried out on ethyl acetate and n-hexane extracts of basil leaves (Ocimum basilicum L.) to assess their anti-biofilm potential in inhibiting biofilm formation by E. faecalis bacteria. Calcium hydroxide paste (calcipex) was used as a positive control, as shown in Table 1.

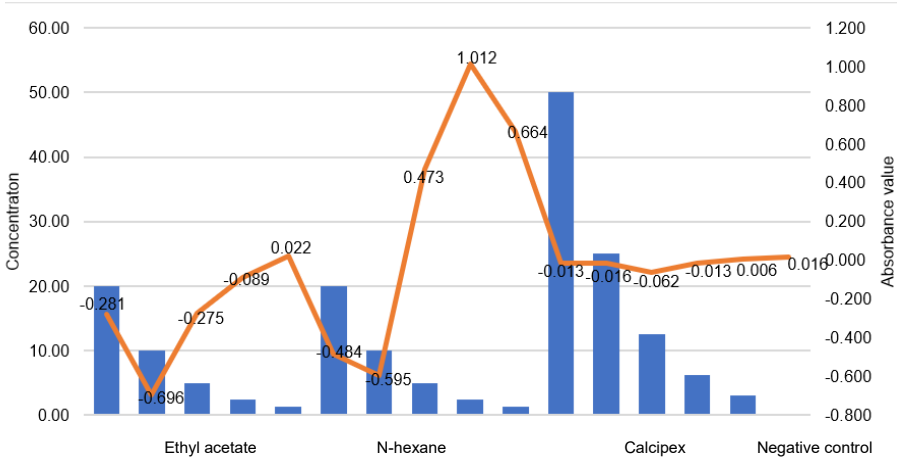

Figure 1. The correlation parameters in determination of biofilm biomass inhibition values affected by addition of ethyl acetate and *n*-hexane extract of *O. basilicum* against *E. faecalis* biofilms formation

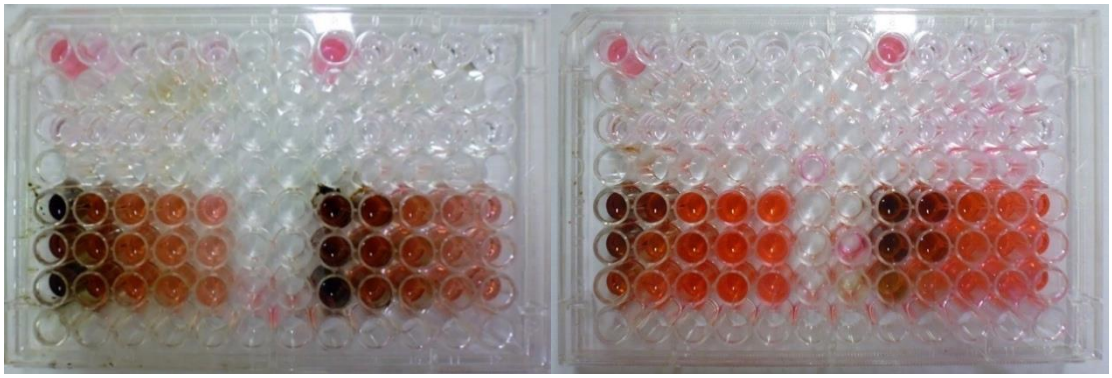

Figure 2. MBIC test of ethyl acetate fraction (left); MBIC test of N-Hexane fraction (right)

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Table 2. Eradication biofilm activity of Ocimum basilicum extracts against E. faecalis biofilm formation

Groups	Concentration (mg/ml)	Absorbance			Average	Eradication (%)
		OD I	OD II	OD III		
Ethyl Acetate extract	20	O/R	O/R	O/R	-	-
	10	-0.237	-0.304	-0.169	-0.237	100
	5	-0.071	-0.021	-0.121	-0.071	100
	2.5	0.164	0.171	0.156	0.164	-
	1.25	0.928	0.898	0.957	0.928	-
n-Hexane extract	20	O/R	O/R	O/R	-	-
	10	O/R	O/R	O/R	-	-
	5	O/R	O/R	O/R	-	-
	2.5	-0.028	-0.222	0.028	-0.074	100
	1.25	0.142	0.080	0.215	0.146	-
Calcipex	50	-0.009	-0.039	-0.034	-0.027	100
	25	0.006	0.006	-0.004	0.003	95.86
	12.5	0.015	0.014	0.018	0.016	75.67
	6.25	0.040	0.035	0.042	0.039	39.44
	3.13	0.019	0.020	0.017	0.019	71.01
Negative control	Without treatment	0.035	0.035	0.035	0.035	-

OD: Optical Density (Turbidity absorbance value); O/R: cannot be read

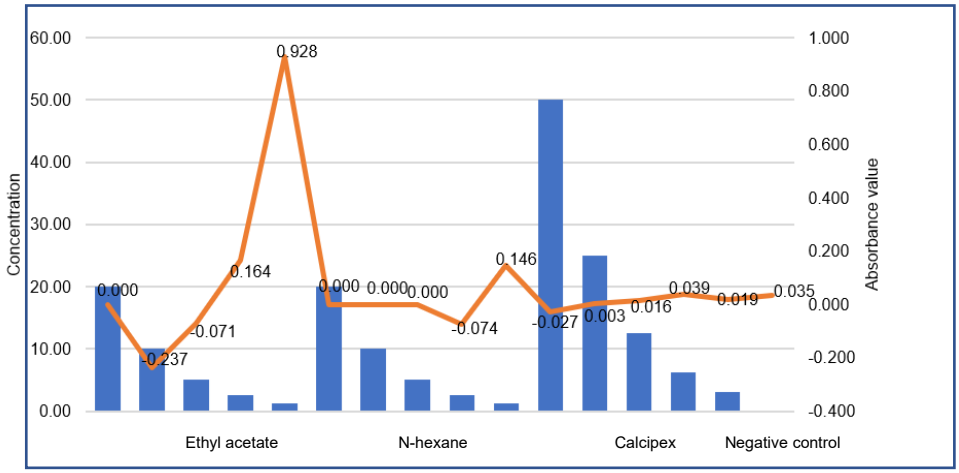

Figure 3. The average of eradication biofilm activity of *Ocimum basilicum* extracts against *E. faecalis* biofilm formation

As presented on the assay data of MBIC in Table 1, the ethyl acetate extract exhibited higher activity than n-hexane extract in inhibiting E. faecalis biofilm formation, with MBIC values of 100% at concentrations of 20, 10, 5, and 2.5 mg/mL. The n-hexane extract inhibited biofilm formation of 100% at two assay concentrations of 20 and 10%.

Moving to the MBIC values of calcipex as positive controls, the assay data showed that the activity of ethyl acetate at low concentration was more active than calcipex with MBIC values

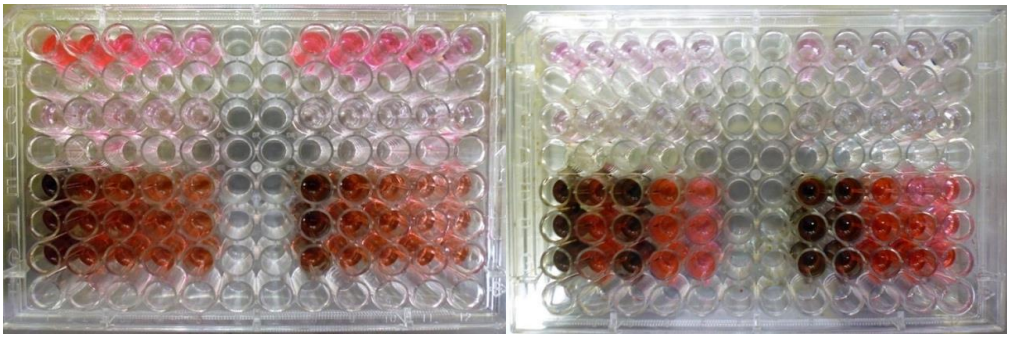

Figure 4. MBEC test of ethyl acetate fraction (left); MBIC test of N-Hexane fraction (right)

of 100% at a concentration of 2.5 mg/mL, while the calcipex showed MBIC values of 75.21% at a concentration of 3.13 mg/mL. As shown in Figure 1, the correlation of parameters in determining the inhibition values of biofilm biomass was affected by the addition of ethyl acetate and n-hexane extract of Ocimum basilicum and calcipex as a positive control in inhibiting the growth of E. faecalis biofilms. The results of the MBIC test are displayed in Figure 2.

As revealed in Figure 1, ethyl acetate extract of 2.5% inhibited the growth of *E. faecalis* biofilm at an average absorbance value of -0.089 compared to negative controls. On the other hand, the *n*-hexane extract of 10% inhibited the growth of *E. faecalis* biofilm formation with an average absorbance value of -0.595 compared to negative controls. Furthermore, a positive control of calcipex at 3.13% reduced the growth of *E. faecalis* biofilm formation at average absorbance value of 0.006 compared to negative controls.

Further analysis was conducted to determine the antibacterial activity. In this analysis, the ethyl acetate and n-hexane extracts were subjected for MBEC assay with calcium hydroxide paste (calcipex) as positive controls on the growth of E. faecalis biofilms. As shown on the assay data of MBEC values in Table 2, the n-hexane extract was active with MBEC value of 100% at the concentration of 2.5 mg/mL, while the ethyl acetate extract was also active with MBEC values of 100% at concentrations of 10 and 5 mg/mL. This finding suggests that n-hexane extract was more active to eradicate of E. faecalis biofilm than ethyl acetate extract.

According to the MBEC values of calcipex as positive controls, the assay data showed that n-hexane extract at low concentration was more active than calcipex with MBEC values of 100% at a concentration of 2.5 mg/mL, while calcipex showed MBIC values of 71.01% at a concentration of 3.13 mg/mL.

The parameter correlation in the determination of biofilm biomass eradication values was affected by the addition of ethyl acetate and *n*-hexane extract of *Ocimum basilicum* and calcipex as a positive control in inhibiting the growth of *E. faecalis* biofilms (Figure 3). The results of the MBIC test can be seen in Figure 4.

Figure 3 shows that at a concentration of 5%, the ethyl acetate extract destroyed the growth of *E. faecalis* biofilms at an average absorbance value of -0.071, while the *n*-hexane extract destroyed the growth of *E. faecalis* biofilms at a concentration of 2.5% with average absorbance value of -0.074. On the other hand, the positive control (calcipex) destroyed the growth of *E. faecalis* biofilms at a concentration of 3.13% with an average absorbance value of 0.019.

The evaluation of assay data by ANOVA statistical test showed differences in antibacterial effectiveness activities between the ethyl acetate and *n*-hexane extracts of *O. basilicum* with calcipex as positive controls against the formation of *E. faecalis* biofilm. Further analysis using the post hoc Tukey test with a value of $p < 0.01$ showed that the ethyl acetate extract was more effective as an antibacterial agent against *E. faecalis* biofilm formation compared to the *n*-hexane extract.

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DISCUSSION

Root canal infections in primary teeth can extend to reach periapical tissue and interfere with the growth and development of permanent replacement teeth.[23] The purpose of performing root canal treatment in primary teeth is to eliminate or eradicate endodontic infections and prevent premature loss of deciduous teeth.[23] Endodontic infections are caused by bacteria in the root canal system which form a biofilm community that is difficult to remove only by instrumentation.[4] Many variables could alter oral environment such as the presence of restorations,[24] or fixed appliances.[25] Therefore, these factors should be taken into careful consideration and future investigations on this topic are needed.

In this research, an environment resembling conditions in the root canal was created by growing *E. faecalis* ATCC 29212 biofilm in a microplate-96 well to further test the effectiveness of *O. basilicum*'s antibacterial activity by looking at MBIC and MBEC from each faction. The results of the test displayed an absorbance value that was read using a microplate reader at a wavelength of 490 nm.[26] This absorbance value shows the biofilm biomass value; the higher the absorbance value, the higher the biomass biofilm value.[27–29]

The MBIC of the ethyl acetate fraction, the *n*-hexane fraction of *Ocimum basilicum,* and calcipex were calculated based on the absorbance value of *E. faecalis* biofilm (Table 1). The results of the test showed that MBIC of *O. basilicum* ethyl acetate fraction at a concentration of 2.5% achieved 100% percent inhibition, effectively suppressing the growth of *E. faecalis* biofilm. Meanwhile, MBIC of *n*-hexane fraction *O. basilicum* at a concentration of 10% exhibited 100% percent inhibition against *E. faecalis* biofilms. Furthermore, calcipex as a positive control with MBIC of 3.13% showed a percent inhibition of 75.21%, proving its ability to inhibit the growth of *E. faecalis* biofilms.

The following are the results of the analysis of the MBEC ethyl acetate fraction and *Ocimum basilicum*'s *n*-hexane fraction. MBEC ethyl acetate fraction of *O. basilicum* at a concentration of 5% with an eradication rate of 100% could inhibit the growth of *E. faecalis* biofilm. MBEC of *n*-hexane fraction of *O. basilicum* observed at a concentration of 2.5% with an eradication rate of 100% effectively stopped the growth of *E. faecalis* biofilms. Additionally, MBEC of calcipex as a positive control exhibited a concentration of 3.13% with an eradication rate of 71.01%, and similarly, it was able to stop the growth of *E. faecalis* biofilms.

The increase in biofilm biomass compared to negative control could be attributed to a bacterial adaptive stress response to antibacterial compounds and the difficulty of penetrating antimicrobial agents into the biofilm.[26] This also suggests that the fraction of *O. basilicum* at low concentrations is unable to penetrate the exopolysaccharide matrix layer which is the main protective layer of biofilm. Other causes include differences in growth rates and nutrients contained outside the surface and in biofilms. For example, the *n*-hexane fraction, known for its non-polar properties, is unable to dissolve in water that has a high oxygen content. This is in line with Macia's research, which reported that some antibacterial agents were active only in aerobic or anaerobic conditions, while biofilms have both of these conditions. The outermost layer of biofilm is aerobic, while the inner layer is anaerobic. Non-polar antibacterial agents, such as n-hexane fraction, are not expected to be able to penetrate the outer layer of biofilm at low concentrations; therefore, higher concentrations are needed. Another reason might be due to the presence of "persister" phenomenon, namely the emergence of dormant variants in biofilms that are tolerant of antibacterial agents.[30,31]

The decrease in biofilm biomass, as indicated by negative absorbance values, indicates that the biomass of *E. faecalis* biomass was lost and the absorbance value read was the absorbance value of the *O. basilicum* fraction. Percentage fluctuations in biofilm inhibition were estimated because at high concentrations the *O. basilicum* fraction was highly saturated, resulting in errors when reading with the microplate reader. The high concentration fraction gives a low average optical density (OD)

value and a high percentage of biofilm inhibition. The results of this study are consistent with those of Rachmawati et al., which found that the greater the extract concentration, the smaller the OD or the absorbance value of biofilm turbidity.[32] Other research conducted by Wangi also confirmed that the higher the concentration of antibacterial material, the lower the OD value and the higher the percentage of inhibition obtained. This condition might be because the greater the concentration of the antibacterial content in the extract will also be greater percentage of biofilm inhibition.[33]

The results of antibacterial power tests on biofilms in this study are also in line with research conducted by Astuti et al. and Khot et al. which reported that *O. basilicum* extract was able to inhibit the growth of oral pathogenic bacterial biofilms.[34,35] The antibacterial power of basil leaves (*O. basilicum*) in this study was estimated due to the content of secondary metabolites it possessed, including phenolic compounds, flavonoids, steroids, triterpenoids, saponins, sterols, tannins, and various other active compounds.[14,36,37] Basil leaves generally contain various compounds such as linalool, estragole, methyl eugenol, 1,8-cineole, and others. Based on GC-MS analysis, linalool is a terpenoid alcohol that is known to have bactericidal (bacterial) and bacteriostatic (inhibit bacteria) abilities. Astuti et al. found that this inhibitory activity occurs through the mechanism of bacterial protein denaturation. According to Greay and Hammer, monoterpenes such as linalool can disrupt the integrity and function of cell membranes, change membrane potential, cause loss of cytoplasmic material, and inhibit the microbial respiratory chain.[38,39]

This study shows that the ethyl acetate fraction of *O. basilicum* has more effective antibacterial power compared to the *n*-hexane fraction. This is in line with research conducted by Turahman[40] and Rasmi et al,[41] which reported that ethyl acetate fraction of basil leaves was able to inhibit the growth of test bacteria better than the *n*-hexane fraction. This is because the ethyl acetate fraction has more antibacterial active compounds as the concentration increases. As a result, the ability of the fraction to inhibit bacterial growth is also better. Alkaloid compounds have also been confirmed to be present in ethyl acetate fraction and ethanol fraction of basil leaves. Anwari reported that compounds dissolved in ethyl acetate solvents include alkaloids and flavonoids. This is in line with Louis's research,[42] which revealed that ethyl acetate fraction of basil, which is semi-polar, can search for secondary metabolites of flavonoids, saponins, tannins and terpenoids, while non-polar *n*-hexane can find secondary metabolites of flavonoids, saponins, and tannins. These compounds can interfere with the integrity of the bacterial cell membrane, causing bacterial cell lysis, thereby preventing biofilm formation. Bacteria need calcium ions in the process of plasma coagulation to form biofilms. The tannin compound in *O. basilicum* can reduce calcium ions and inhibit the process of plasma coagulation, ultimately preventing the formation of biofilm formation.[32]

In this study, the entire extract partition from the basil (*O. basilicum*) leaves was used; therefore, it remains unclear which content plays a significant role as an antibiofilm agent in each *O. basilicum* fraction. Further research is needed to find the effective concentration that can be applied as an alternative in root canal medicaments.

CONCLUSION

The results of this study showed that the ethyl acetate and *n*-hexane fractions of basil leaves (*O. basilicum*) have the ability to inhibit and eradicate *E. faecalis* ATCC 29212 biofilm in vitro with different antibacterial effectiveness. The ethyl acetate fraction of *O. basilicum* exhibited more effective antibacterial ability compared with the *n*-hexane fraction against *E. faecalis* ATCC 29212 biofilm in vitro. Further research is needed to investigate the use of *O. basilicum* as an alternative to root canal medicaments in primary teeth. The edible kemangi herb (*O. basilicum*) can be used as an alternative natural treatment for dental diseases caused by oral bacteria. The results of this study are important for future research in isolating the

active substance of antibiofilms and determining their activity in silico, in vivo and clinical trials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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