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Original article

Antibiofilm activity of neem leaf (*Azadirachta indica* A. Juss) ethanolic extracts against *Enterococcus faecalis* in vitro

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ABSTRACT

Background: *Enterococcus faecalis commonly infects root canals by forming a biofilm. Extracts from neem leaves (Azadirachta indica A. Juss) have been shown to have antibacterial properties, indicating their potential in preventing or treating biofilm formation caused by bacteria.* ***Purpose:*** *This study aims to investigate the phytochemical compounds present in neem leaves (Azadirachta indica A. Juss) and establish the concentration of ethanol-based neem leaf extract that can effectively inhibit the in vitro growth of Enterococcus faecalis biofilm.* ***Methods:*** *This study employed the maceration technique for extraction, gas chromatography mass spectroscopy for the analysis of plant chemicals, and a microtiter plate assay for measuring biofilm formation with treatment concentrations of 6.25%, 12.5%, 25%, 50%, and 75%, with a positive control of 0.2% chlorhexidine.* ***Results:*** *A phytochemical analysis revealed that the ethanol extract of neem leaves contained 22 different metabolites, mainly terpenoids and fatty acids. The extract demonstrated antibiofilm activity only at a concentration of 12.5% with an average biofilm inhibition of 36.85%. However, lower concentrations of 6.25%, 25%, 50%, and 75% had the opposite effect, promoting biofilm formation in Enterococcus faecalis.* ***Conclusion:*** *Phytochemical metabolite contained in the ethanolic extracts of neem leaves might contribute a promising agent in treating a biofilm-mediated root canal infection of Enterococcus faecalis.*

Keywords: *Antibiofilm; Azadirachta indica A. Juss; Enterococcus faecalis; ethanol extract*
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INTRODUCTION

Root canal infections are generally caused by microorganisms. The presence of microorganisms penetrating dental pulps followed by the colonization of the root canal system is a major cause of periapical pathology that leads to the failure of root canal treatment.[1] One of the bacterium often isolated in root canal treatment is *Enterococcus faecalis (E. faecalis).* It is a group of gram-positive enterococci that have a cocci shape and are classified as lactic acid bacteria that can be found in the root canals of teeth along with other pathogens, including *Streptococcus, Actinomyces, Staphylococcus*, and *Lactobacillus*.[2]

Root canal treatment aims to repair the infected tooth by restoring the infected tissue, eliminating the pathogenic bacteria, and preventing recontamination after root canal treatment.[3] The pathogenic bacteria causing root canal infections are developing resistance, which poses major challenges in treatment. *E. faecalis* can survive in a variety of environmental conditions, including environments with extreme alkaline pH;[4] they remain in root canals after disinfection using sodium hypochlorite and chlorhexidine (CHX).[5] Furthermore, root canal infections may be difficult to treat due to bacterial infiltration into the dentin, accompanied by virulence factors and biofilm production. Virulence factors with the ability to form biofilms, such as gelatinase production, limit the penetration of antibiotics to achieve effective concentrations at the target site, which hinders the healing process.[6]

A biofilm is a cooperative association of microorganisms that adhere to surfaces, biotic and abiotic, using extracellular polymeric substances (EPS) and glycocalyx, and they communicate through a quorum sensing (QS) system.[7] Biofilms forming in the intracanal and periapical dentin

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allow *E. faecalis* to be more resistant to phagocytes, antibodies, and antibiotics compared with bacteria that do not form biofilms.[8] In vitro studies have shown different stages of *E. faecalis* biofilm development on root canal dentin.[9]

Increasing bacterial resistance to drugs requires efforts to find antimicrobial agents that are effective against pathogenic bacteria. Alternative treatments to overcome bacterial resistance due to the formation of biofilms include searching for natural compounds derived from plants that are able to inhibit the formation of biofilms, like neem leaves (*Azadirachta indica* A. Juss). The neem plant has long been used to maintain oral hygiene and prevent cavities as well as prevent gingival disease and periodontitis. Neem leaves have several active compounds in the form of alkaloids, tannins, essential oils, and flavonoids that have potential as antimicrobials.[10] Previous research reported that neem leaves have antibiofilm properties, which strengthen the possibility of using neem extract in eradicating biofilm-mediated infections.[11] Certain concentrations of neem extract show a significant reduction in the number of exopolysaccharides, changing the biofilm structure, which could facilitate the penetration of antibiotics into the bacterial community.[12] Therefore, this study aims to identify the phytochemical compounds present in neem leaves (*Azadirachta indica* A. Juss) and determine the concentration of the ethanol extract of neem leaves that can effectively prevent the in vitro formation of biofilm by *E. faecalis*.

MATERIALS AND METHODS

The extraction was conducted following the method previously described.[13] Neem leaves (*Azadirachta indica* A. Juss) were collected from Limpok, Darussalam, Aceh Besar, Indonesia. A total of 2 kg of fresh neem leaves were washed thoroughly and dried at room temperature. Dried neem leaves were mashed using a blender, filtered to get a finer powder, and then weighed. The neem leaves were then extracted using the maceration method using 96% ethanol as a solvent, and the ratio between ethanol and the Simplicia solvent was 1:10. The initial stage of maceration was carried out by soaking Simplicia in 7 liters (L) of 96% ethanol solvent (7/10 of 10 L) for five days. The container used for maceration was covered with aluminum foil and stored in a location shielded from the sun. After that, filter paper was utilized to separate the filtrate and dregs. The dregs were again immersed in 3 L of 96% ethanol (3/10 of 10 L) for five days. The mixed extract and solvent were stirred occasionally every day. Extract yield was calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Extract weight}}{\text{dried Simplicia weight}} \text{x } 100\%$$

The analysis of the phytochemical compounds in the ethanol extract of neem leaves was performed using gas chromatography mass spectroscopy (GC/MS) equipment (Shimadzu GCMS-QP 2010 Ultra) with reference to the method developed by a previous study.[14] Sample preparation was carried out by diluting 1 g of the sample in ethanol with a ratio of 1:2. Moreover, 5 µL of the sample was injected into the GC/MS system in splitless mode. The stationary phase used in this study was Rxi-1ms (100% dimethyl polysiloxane), which had a column length of 30 mm and a diameter of 0.25 mm. The carrier gas used was helium conditioned at a pressure of 37.1 kPa and a flow rate of 0.72 ml/min. The injector temperature, the ion source temperature, and the surface temperature were set at 250°C, 230°C, and 230°C, respectively, and the split mode was 10. The column temperature used was 70°C–270°C, with an increase program as follows: the temperature was increased from 70°C to 230°C, with an increase rate of 10°C /minutes, and then held for three minutes before finally being raised again until it reached a final temperature of 270°C.

Bacterial cultures of *E. faecalis* were grown on agar plates and broth media. The bacterial culture was inoculated on a tryptone soya agar (TSA) medium, which was then incubated at 37°C for 24 hours. Furthermore, isolates were inoculated from the TSA medium into a tryptone soya broth (TSB) media containing 2% sucrose in a volume of 50 mL, followed by a 24-hour incubation at room temperature on an orbital shaker.[15]

Antibiofilm assay for *E. faecalis* was carried out using the microtiter plate biofilm essay method.[16] A suspension of *E. faecalis* in a 2% TSB sucrose medium was prepared. A 25 µl bacterial suspension was then inoculated into each well on a round bottom microplate. The ethanolic extract of neem leaves was diluted in 2% TSB sucrose media to obtain concentrations of 6.25%, 12.5%, 25%, 50%, and 75% (w/v). A 0.2% CHX was used as a positive control. A 100 µl of each series concentration of neem leaf ethanol extract was added to the wells containing the bacterial suspension. A 100 µl 0.2% CHX was also added as a positive control well, whereas, in the negative control, no extracts nor CHX were added. The microtiter plate was then incubated for 72 hours at 37°C. The microplate was then washed three times using a sterile 200 µl of phosphate buffered saline. The washed microplate was then added with 200 µl of 96% ethanol for 15 minutes before it was drained and dried. A 200 µl of 0.1% crystal violet was then added to the dry microplate for 15 minutes before it was washed using sterile distilled water three times and dried for several minutes. A 125 µl of 30% glacial acetic acid was added and allowed to stand for 15 minutes. A total of 125 µl of 30% glacial acetic acid was then transferred to a new flat bottom microplate to determine its optical density (OD) using a microtiter plate reader at a wavelength of 570 nm, and the biofilm inhibition was then calculated using the following formula:

$$\text{\% inhibition} = \frac{\text{OD negative control} - \text{OD experiment}}{\text{OD negative control}} \text{x } 100\%$$

Quantitative data was collected for the percentage value of the area of the phytochemical component with GC/MS and the OD value of the percentage of antibiofilm.[16] OD values were analyzed using one-way analysis of variance

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(ANOVA) ($P \leq 0.05$ on two-tailed) and further analyzed using the Turkey test to determine the differences in each ethanolic extract concentration of neem leaves. Microsoft Excel and SPSS 18 were used to tabulate and analyze the data.

RESULTS

The dried neem leaves were extracted using the maceration method with 96% ethanol as a solvent. The ethanol extract of neem leaves produced from 1 kg of Simplicia was 114.63 g with a total extract yield of 11.46%. The resulting extracts were dark green with a thick consistency.

The GC/MS analysis showed that there were 22 phytochemical compounds detected from the ethanol extract of neem leaves in this study. The detected phytochemical compounds were characterized by the presence of 22 eluted peaks starting from the fourth minute to the 29th minute (Figure 1). The detected compounds generally belong to the group of alkaloids, acetals, terpenoids, and fatty acids that have an area between 0.41% and 45.33% (Table 1). Phytol; (E)-9-octadecenoic acid ethyl ester; and hexadecanoic acid, ethyl ester (CAS) ethyl palmitate were the three most phytochemical compounds in the ethanolic extracts of the neem leaves in this study. They accounted for 45.33%, 8.35%, and 7.97%, respectively. Phytol compounds had the highest peak, with an area of 45.33% and retention of 20.973 minutes, whereas (E)-9-octadecenoic acid ethyl ester had an area of 8.35% and a retention time of 21.362 minutes. The ethyl ester (CAS) ethyl palmitate detected had an area of 7.97% and a retention time of 19.746 minutes.

The results of the antibiofilm test of neem leaf ethanol extract against *E. faecalis* bacteria using the microtiter plate biofilm assay method showed the potential to inhibit the formation of biofilms. The OD value of the percentage of antibiofilm obtained was analyzed using the one-way ANOVA test ($P < 0.05$), which showed a significant difference in the OD value of each treatment with the concentration of neem leaf ethanol extracts. Further tests using the Turkey analytical test were carried out to determine the concentration of neem leaf ethanol extract that has potential as an antibiofilm compared with the positive control (0.2% CHX). It is evident that only the ethanolic extract of neem leaves at a concentration of 12.5% showed significant antibiofilm activity of 36.85% (Table 2). Table 2 also shows that the concentration of 12.5% has a relatively high average percentage of antibiofilm compared with concentrations of 6.25%, 25%, 50%, and 75%. The positive control (0.2% CHX) showed a higher percentage of antibiofilm than the concentration of 12.5%, which was 68.28%. There was no significant difference between the ethanolic extracts of neem leaves at a 12.5% concentration and the positive control.

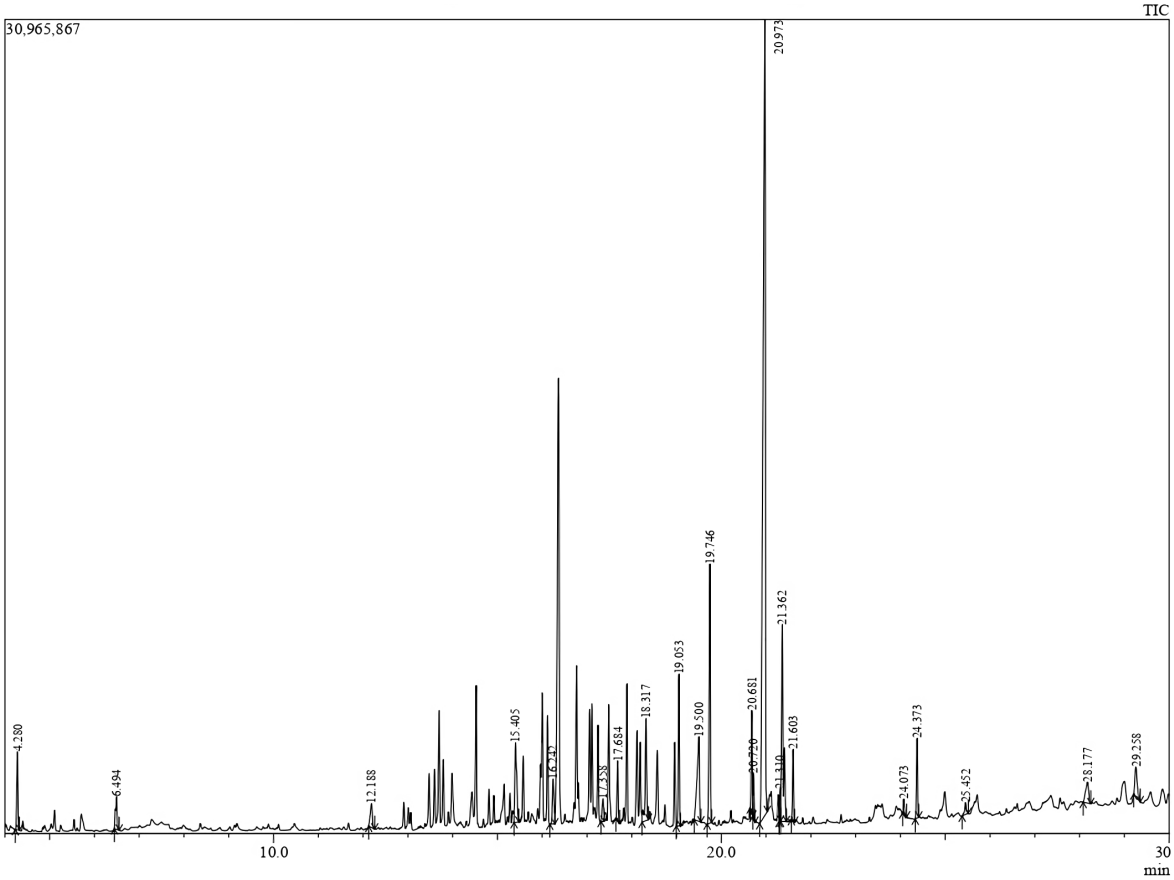

Figure 1. Chromatogram of GC/MS analysis of neem leaf (*Azadirachta indica* A. Juss) ethanol extracts.

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Table 1. The GC/MS analysis of phytochemical compounds in the ethanolic extracts of neem leaves (*Azadirachta indica* A. Juss)

Peak#	Retention Time (min)	Area (%)	Compound Name	Chemical Formula	Molecular Weight (g/mol)
1	4.28	2.18	Pyrrolidine-2,2,5,5-d4	C4H5D4N	71
2	6.494	1.25	1,1-Diethoxypentane	C9H20O2	160
3	12.188	1.18	1-(1-Hydroxy-1-methyl-ethyl)-cyclobutanecarboxylic acid	C8H14O3	158
4	15.405	3.64	(-)-Caryophyllene oxide	C15H24O	220
5	16.242	1.78	Epiglobulol	C15H26O	222
6	17.358	0.86	Tetradecanoic acid (CAS) myristic acid	C14H28O2	228
7	17.684	1.6	Tetradecanoic acid, ethyl ester (CAS) ethyl myristate	C16H32O2	256
8	18.317	3.72	9-Hexadecenoic acid, phenylmethyl ester, (Z)-	C23H36O2	344
9	19.053	4.25	Hexadecanoic acid, methyl ester	C17H34O2	270
10	19.5	5.03	n-Hexadecanoic acid	C16H32O2	256
11	19.746	7.97	Hexadecanoic acid, ethyl ester (CAS) Ethyl palmitate	C18H36O2	284
12	20.681	2.73	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C19H32O2	292
13	20.72	0.95	9-Octadecenoic acid, methyl ester, (E)-	C19H36O2	296
14	20.973	45.33	Phytol	C20H40O	296
15	21.31	0.92	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C19H32O2	292
16	21.362	8.35	(E)-9-Octadecenoic acid ethyl ester	C20H38O2	310
17	21.603	1.87	Octadecanoic acid, ethyl ester	C20H40O2	312
18	24.073	0.41	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	C19H38O4	330
19	24.373	2.25	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS) Bis(2-ethylhexyl) phthalate	C24H38O4	390
20	25.452	0.5	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C21H40O4	356
21	28.177	1.55	Oxalic acid, hexadecyl 1-menthyl ester	C28H52O4	452
22	29.258	1.68	Lanosterol	C30H50O	426

Table 2. Average antibiofilm percentage of neem leaf (*Azadirachta indica* A. Juss) ethanol extract against *Enterococcus faecalis*

Treatments	Antibiofilm activity (%) ± SD		
6.25%	-29.06[c]	±	27.66
12.5%	36.85[d]	±	5.53
25%	-106.29[b]	±	24.53
50%	-110.19[b]	±	8.43
75%	-287.28[a]	±	29.197
Positive control (0.2% CHX)	68.28[d]	±	2.702

Notes: a, b, c, and d's different notations show a significant difference at the 95% confidence interval.

DISCUSSION

The search for natural-based compounds that can be applied as an antibiofilm to overcome biofilm-related infections like root canal diseases is increasing. This study elucidates the phytochemical compounds of ethanolic extracts of neem leaves (*Azadirachta indica* A. Juss) and shows their potential as an antibiofilm against *E. faecalis*. The extraction efficiency using 96% ethanol as a solvent in this study was quite high (i.e., 11.46%). This result is higher than other studies demonstrating a total extract yield of 9.75%[17] or 3.5%.[18] The different percentage values of the total extract yield indicate how large the amount of phytochemical content is in Simplicia. The outcome of the chemical extraction process is influenced by the choice of solvent, duration of the extraction, temperature, ratio of the sample to solvent, and the chemical and physical properties of the sample.[19] The variety in extraction efficiency is also attributed to differences in the polarity of compounds present in plants. The yield of extracts with high phytochemical compounds can be produced with the right maceration time. Phytochemical compounds do not completely dissolve into the solvent if the maceration time is too short and will be degraded if the maceration time is too long.[20] The high extract yield value was also influenced by the size of the dried Simplicia leaves. Simplicia with a small size can increase the surface area so that more contact with the solvent occurs during the soaking process.[21]

Based on the GC/MS analysis, the phytochemical components contained in the ethanolic extract of neem leaves in this study were primarily terpenoid and fatty acid groups. Phytol, (E)-9-octadecenoic acid ethyl ester, and hexadecanoic acid, ethyl ester (CAS) ethyl palmitate were detected as the three most phytochemical compounds in the ethanolic extracts of the neem leaves in this study. Phytol is a compound of the diterpenoid group, whereas

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(E)-9-octadecenoic acid ethyl ester and hexadecanoic acid, ethyl ester (CAS) ethyl palmitate are compounds of the fatty acid group. Previous investigations found the presence of alkaloids, terpenoids, flavonoids, tannins, saponins, glycosides, and phenolics in the phytochemical screening results of neem leaves.[22] Internal and environmental factors can influence the diverse secondary metabolite composition of a plant. Internal parameters are determined by genetic material, while external factors include nutrient content, altitude, temperature, light intensity, humidity, and pH. The observed differences in the presence of phytochemicals can also be attributed to differences in solvent polarity.[17]

Phytol (3,7,11,15-tetramethyl-2-hexadecen-l-ol) is a type of diterpenoid that acts as a plant metabolite and can be found in vitamins K and E, as well as other forms of tocopherols. Previous studies have demonstrated that *phytol* possesses antibacterial properties against *Clostridium sporogenes, E. faecalis*, and *Sarcina lutea*.[23] This organic compound showed a greater ability to inhibit the growth of *E. faecalis* (with a minimum inhibitory concentration (MIC) of less than 2 g/mL) compared with gentamicin and ampicillin (MIC of 5 and 16 g/mL respectively). *Phytol* also has the ability to reduce biofilm thickness, alter the shape of the biofilm, and significantly inhibit the production of EPS in *Serratia marcescens*.[24]

Fatty acids are reported to have antibacterial and antibiofilm effects. Palmitic acid and stearic acid have antibacterial activity of vancomycin-resistant *E. faecalis* with MICs of 2 g/ml and 0.5 g/ml, respectively.[25] There are two molecular mechanisms that can explain the antimicrobial activity of fatty acids, namely changes in biochemical functions and the loss of viability through specific interactions with sites within microorganisms or disruptions in the structure of microorganisms through nonspecific interactions, inhibiting the physiological functions of microorganisms.[26]

The ethanolic extract of neem leaves at a concentration of 12.5% showed the presence of antibiofilm activity (Table 2). Previously, studies reported the effectiveness of neem leaf extract against *Streptococcus sanguis* biofilm in the oral cavity by reducing the plaque index and bacterial count significantly compared with the control group.[27] Moreover, another study using neem leaf extracts showed biofilm inhibition, a decrease in the percentage of hydrophobicity, and a decrease in the adhesion ability of *Pseudomonas aeruginosa* using neem leaf extract.[28]

Antibiofilm activity against *E. faecalis* bacteria could be caused by the phytochemical content contained in the ethanolic extract of neem leaves, such as terpenoids, fatty acids, and alkaloids. Terpenoids are able to damage planktonic cells in biofilms and disrupt the integrity of bacterial cell membranes.[29] Medium-chain fatty acids resemble QS molecules, and they have antibiofilm activity by inhibiting QS signaling.[30] Alkaloids with a small composition in the ethanolic extract of neem leaves are also thought to have an antibiofilm role. Alkaloids can effectively eliminate biofilms and inhibit their formation (antibiofilm activity) on both gram-positive and gram-negative bacteria.[31]

The concentration of neem leaf extract that was lower than 12.5% (i.e., 6.25%) had an average antibiofilm percentage of -29.06%. This shows that low concentrations cannot inhibit the formation of biofilms. The lack of antibiofilm properties of neem leaf extracts in the test could be caused by the low concentration of bioactive compounds at low concentrations. Similar findings found in other investigations signify increased biofilm development in *Pseudomonas aeruginosa* 27853 with the addition of neem extracts.[32] Furthermore, other findings showed that the clinical isolate of *Pseudomonas aeruginosa* in sub-MIC of biocidal agents, such as CHX, savlon, and deconex, showed biofilm induction.[33] Moreover, antibiotics might induce biofilm formation, particularly at low concentrations, as the drugs trigger a bacterial cell response to environmental stress that has a role in bacterial protection.[34]

The ethanol extract of neem leaves at higher concentrations of 25%, 50%, and 75% did not show any antibiofilm activity but were thought to induce the formation of *E. faecalis* biofilms, with an average antibiofilm percentage of -106.29%, 110.19%, and -287.28%, respectively. There was no significant difference between ethanolic extracts of neem leaves and the positive control. A 0.2% CHX was used as a positive control because it is the gold standard of root canal irrigation solutions with the ability to degrade bacterial biofilm.[35] Both extract concentrations and solubility might contribute to this biofilm formation induction. The use of more concentrated extracts is complicated by the low solubility of the components as the extract becomes viscous and difficult to dissolve at high concentrations.[32] Other studies added that the inhibitory power of the extracts against bacteria was not proportional to the increase in the concentration of the extract.[36] The high concentration of ethanol extract causes an increase in its viscosity that affects the rate of diffusion of antibiofilm compounds on bacteria. An insoluble extract can promote the development of a thicker biofilm, as the biofilm thickness is influenced by the number of solid particles in the solution.[37] The presence of bis-(2-ethylhexyl) phthalate, which is a plasticizer, in neem leaf extract is thought to cause the extract solution to be less soluble in high concentrations since di-(2-ethylhexyl) phthalate on bacterial biofilms increased the production of EPS, providing functional and structural integrity to biofilms, which determine their physicochemical properties.[38]

Overall, it can be concluded that the ethanolic extract of neem leaves has an effect on inhibiting the formation of the biofilms of *E. faecalis* bacteria at a concentration of 12.5%. Phytochemical metabolite contained in the ethanolic extracts of neem leaves might be a promising agent in treating a biofilm-mediated root canal infection of *E. faecalis*. Further studies addressing the potential of specific active compounds from the terpenoid and fatty acid groups in neem leaves for biofilm inhibition need to be conducted.

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