

ANTIBACTERIAL EFFECT OF XANTHORRHIZOL (*CURCUMA XANTHORRHIZA* ROXB.) AGAINST THE BIOFILM OF *FUSOBACTERIUM NUCLEATUM*

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ABSTRACT

Objective: Endodontic infections are treated with a root canal; one of the stages involves using an irrigation solution to eliminate microorganisms. Sodium hypochlorite (NaOCl) is an irrigation solution used to eliminate *Fusobacterium nucleatum*, the common cause of primary endodontic infections. However, this material is synthetic and can cause side effects and inflammation of the periapical tissues. Thus, an irrigation solution, such as xanthorrhizol, which is made of natural ingredients, is required. However, xanthorrhizol's antibacterial effect against *F. nucleatum* has never been assessed. Thus, this study aimed to analyze the effect of xanthorrhizol, derived from *Curcuma xanthorrhiza* Roxb., against *F. nucleatum* ATCC 25586 biofilm.

Methods: The methylthiazol tetrazolium (MTT) assay test and colony count test were performed to assess *F. nucleatum* eradication after exposure to xanthorrhizol at various concentrations (0.5%, 0.75%, 1%, 1.25%, and 1.5%) and 2.5% NaOCl, which was used as positive control.

Results: Significant differences were observed in terms of cell viability after treatment with xanthorrhizol at concentrations of 1.25% versus 0.5%, 0.75%, and 1%. The MTT assay test was used to evaluate cell viability in the biofilm and cell metabolism activities. Results showed no significant differences in terms of efficacy between xanthorrhizol at concentrations of 1.25% and 1.5%, which are considered effective against *F. nucleatum*, and 2.5% NaOCl ($p > 0.05$).

Conclusion: The effect of xanthorrhizol at concentrations of 1.25% and 1.5% against *F. nucleatum* is similar to that of 2.5% NaOCl.

Keywords: Xanthorrhizol, *Fusobacterium nucleatum*, Sodium hypochlorite, Antibacterials, Biofilm.

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INTRODUCTION

Microorganisms in the root canal cause endodontic infections [1]. In general, various types of anaerobic bacteria dominate these infections. Lee *et al.* (2017) have found that approximately 70.3% and 29.7% of bacteria in the root canal are anaerobes and aerobes, respectively. Some types of anaerobic bacteria, which are found in endodontic infections, are gram-negative bacteria, including *Fusobacterium nucleatum* [2]. Siqueira and Rocas (2011) have used reverse-capture checkerboard DNA-DNA hybridization, and the proportion of *F. nucleatum* bacteria was the highest in symptomatic primary endodontic infections (acute apical abscess; $>10^6$ CFU). Furthermore, it was the fourth highest bacteria population (as many as 10^6 CFU) in cases of asymptomatic primary endodontic infections [3]. Pereira *et al.* (2017) conducted a study using real-time polymerase chain reaction on the teeth with periapical lesions caused by endodontic failure. Their results showed that, in the area around the tip of the root and periapical tissue with chronic lesion, the percentage (71.3%) of *F. nucleatum* was the highest, followed by *Dialister pneumosintes* (58.3%) and *Tannerella forsythia* (48.3%) [4].

F. nucleatum is an obligate gram-negative anaerobic bacterium, and it has a role as a bridge bacterium between early and late bacterial colonization and biofilm formation. These bacteria's outer and inner membranes are limited by the periplasmic space, which contains the peptidoglycan layer. The inner layer membranes comprise the phospholipid bilayer; the amount between phospholipids and proteins was balanced. The outer membranes serve as a molecular sieve; they have an asymmetrical shape and are composed of phospholipids, lipopolysaccharide, lipoproteins, and proteins. The lipopolysaccharide (LPS) components on *F. nucleatum* include lipid A and O-antigen polysaccharide, which is a lipopolysaccharide

endotoxin [5,6]. LPS can trigger the synthesis of interleukin 1 alpha and tumor necrosis factor-alpha (TNF- α), which is released from macrophages and is involved in bone resorption in apical periodontitis [7].

Virulence factors make *F. nucleatum* pathogenic to human body tissues [8]. In addition to oral diseases, the bacteria can cause several systematic diseases [9], based on the capability to co-aggregate with other bacterial species in co-infections. Previous research has shown that *F. nucleatum* can co-aggregate with *Enterococcus faecalis*. The interaction between these two bacteria has contributed significantly to the occurrence of endodontic lesions [10]. Reis *et al.* (2016) have conducted *in vivo* studies on mice, and results showed that exposure to *F. nucleatum* biofilms and both *F. nucleatum* and *E. faecalis* could induce higher expressions of RANKL, TNF- α , and interferon-gamma than exposure to *E. faecalis* biofilm on the 10th day [11]. *F. nucleatum* may be found in oral cavity infections, such as post-treatment endodontic diseases, acute dental abscess, endodontic flare-ups, and periodontal disease [4,9].

Successful endodontic treatment is based on eliminating bacteria from the root canal through disinfection [12]. This procedure can be performed through instrumentation, canal irrigation, and root canal treatment. The root canal system has a complex anatomical shape. Thus, the instrumentation process was less effective in eliminating microorganisms. Thus, irrigation during instrumentation is required. Unlike mechanical instrumentation, this method may result in a complete debridement and can reach the entire root canal system [13].

NaOCl is the most common irrigation solution used in endodontic treatment. It has an antibacterial effect and can dissolve necrotic

tissues, organic dentin components, and biofilms. A higher NaOCl concentration can increase these irrigation materials' toxicity levels if it comes in contact with the periapical tissues, and it can trigger an allergic reaction [14]. Considering that the irrigation materials currently used have toxic properties, alternative irrigation solutions that are made from natural materials and have antibacterial properties must be considered.

Indonesia is an agricultural country, with farming areas and large plantations for medicinal plants. Turmeric (*Curcuma xanthorrhiza* Roxb.) is one of the country's natural commodities [15]. It ranked ninth among the best herbal plants that have numerous benefits, including its use in herbal medicine, which is an in-demand commodity. The use of Turmeric (*Curcuma xanthorrhiza* Roxb.) for the manufacture of traditional herbal medicine is very large in number, reaching 3000 dry tons per year [16]. Moreover, turmeric is an herbal medicine component because it has a metabolite content, which contains bioactive compounds produced through a secondary metabolite, which is effective against pathogens, or it plays a role in environmental adaptation. Thus, it used as a component in medicines for humans [17]. Turmeric has hepatoprotective, antioxidant, anti-inflammatory, anticariogenic, antimutagenic, immunomodulatory, anti-aging, antibacterial, antifungal, insecticidal, and anticoagulant properties [18].

Turmeric (*Curcuma xanthorrhiza* Roxb.) has three functions, which are as follows: Starch, curcumin, and essential oils [18]. Essential oils contain xanthorrhizol, which is a major active compound, with a concentration of 64.38% [17]. The active compound of xanthorrhizol helps disrupt oral bacteria's biofilm formation [19]. Rukayadi and Hwang (2006) have reported that this active substance has a strong antibacterial effect against *Streptococcus mutans* [20]. Prijatmoko (2018) has shown that essential oil, extracted from *Curcuma xanthorrhiza* Roxb., can inhibit *F. nucleatum* and *E. faecalis* growth, and the most effective concentration is 100% [21]. According to Kim et al. (2008), xanthorrhizol at a concentration of 1% is considered effective against *S. mutans* biofilms [19].

This study aimed to analyze the antibacterial effect of xanthorrhizol, an active substance in turmeric (*Curcuma xanthorrhiza* Roxb.), against *F. nucleatum* ATCC 25586 biofilms.

MATERIALS AND METHODS

We conducted an *in vitro* laboratory study. The independent variable was the antibacterial effect of xanthorrhizol at various concentrations (0.5%, 0.75%, 1%, 1.25%, and 1.5%) and 2.5% sodium hypochlorite (NaOCl). Meanwhile, the dependent variable was the test material's antibacterial effect against *Fusobacterium nucleatum* ATCC 25586 biofilm.

In this study, 95% xanthorrhizol had been isolated from turmeric (*Curcuma xanthorrhiza* Roxb.) extract purchased from Javaplant. This solution was obtained by extracting ginger through percolation, evaporation, separation, and purification. The percolation process produces *Curcuma simplicia*. The resulting extract is still in the form of ethanol, which contains xanthorrhizol and curcumin, called percolates. Then, the percolate was concentrated through evaporation with a rotary evaporator, until it became a liquid concentrate. Next, xanthorrhizol and curcumin were separated, and this process involved mixing the liquid concentrate with hexane 1:1. Curcumin remained soluble in ethanol and xanthorrhizol in hexane. Curcumin was below and xanthorrhizol was above the solution. The next process was purifying xanthorrhizol. In total, 10 mL of xanthorrhizol was used for each concentration. The solution was diluted with dimethyl sulfoxide (DMSO) to obtain a concentration of 0.5%, 0.75%, 1%, 1.25%, and 1.5%.

In total, 100 µL of *F. nucleatum* ATCC 25586 was mixed into 990 µL of Brain Heart Infusion (BHI) broth (Oxoid, the UK) and was incubated in an anaerobe condition (N₂, 80%; H₂, 10%; and CO₂,

10%) for 72 h at a temperature of 37°C. To assess the biofilm count, the suspension was diluted into 1 × 10⁶ CFU/mL. The biofilms were incubated and transferred to 96-well plates by adding 100 µL of *F. nucleatum* suspension, and the mixture was added to every well plate. Furthermore, the suspension was incubated at 37°C for 72 h. When biofilms had formed in each well, 100 mL of various xanthorrhizol concentrations were added. In the positive control, 100 mL of 2.5% NaOCl was added to each well. In the negative control, the *F. nucleatum* suspension had no intervention. Then, the biofilm was incubated with the test substance for 15 min at a temperature of 37°C. The control blank comprises the BHI broth medium and xanthorrhizol solution at various concentrations. This process was duplicated for each xanthorrhizol concentration and positive control. The methylthiazol tetrazolium (MTT) test was then performed, and the first stage involved rinsing each well plate with 100 mL of phosphate-buffered solution (PBS) solution, and manufacturing MTT solution with a concentration of 5 mg/mL. Moreover, 10 mL of MTT solution was added to each well containing the test material and was incubated for 3 h at a temperature of 37°C. Then, 100 mL of acidified isopropanol was added to each well. The well plate was placed on a shaker for 1 h. The optical density value was assessed using a microplate reader with a wavelength of 490 nm. Biofilm eradication was expressed as a percentage of formula eradication [22,23].

$$\text{Eradication(\%)} = \left(1 - \frac{\left(\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blancsample}}}{\text{OD}_{\text{negativecontrol}} - \text{OD}_{\text{blancnegativecontrol}}} \right) \times 100\%}{1} \right)$$

Next, the antibacterial effect of xanthorrhizol at concentrations of 0.5%, 0.75%, 1%, 1.25%, and 1.5% and 2.5% NaOCl against *F. nucleatum* ATCC 25586 was assessed using the colony count method. In total, 100 µL of the biofilms of *F. nucleatum* ATCC 25586 from stock cultures were taken, placed on a well plate with a predetermined design, and then incubated for 72 h at a temperature of 37°C. Then, the well plate was rinsed to remove planktonic materials. The formed biofilm was exposed to the test material according to the specified plate design.

The samples, which have been treated and remained at the bottom of the plate, were scraped, placed in a PBS solution, and mixed using a vortexer for 20 s; then, 1 µL of the solution was cultured in agar medium. Each sample was dropped and rubbed on the BHI agar medium and then incubated at 37°C for 72 h. The antibacterial effect of all the test materials was evaluated by calculating the colonies. *F. nucleatum* bacteria, which are still alive after exposure to the test material and formed a colony in the BHI preparation, were then calculated manually. The lower the number of colonies formed, the lower the CFU/mL value, and the higher the antibacterial effect.

Data were collected, and the Statistical Package for the Social Sciences software, version 24, was used in all analyses. Statistical analysis was performed to test data normality. If the data had a normal distribution, parametric tests were conducted using one-way analysis of variance. Meanwhile, if the data distribution were abnormal, the Kruskal-Wallis test was used. A p < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The antibacterial effect of xanthorrhizol at various concentrations (0.5%, 0.75%, 1%, 1.25%, and 1.5%) was analyzed using the MTT assay. Table 1 shows the eradication value of xanthorrhizol at various concentrations and 2.5% NaOCl against the biofilms of *F. nucleatum* ATCC 25586.

Table 1 shows there were significant differences between xanthorrhizol at various concentrations and 2.5% NaOCl in the eradication percentage (%) of the *F. nucleatum* ATCC 25586 biofilm.

This study examined the antibacterial effect of *xanthorrhizol* at various concentrations against *F. nucleatum* biofilm. Biofilm formation is one of the most important virulence factors of *F. nucleatum* due to a higher resistance ability to host defense or antibacterial agents compared to *F. nucleatum*'s planktonic forms. Under biofilm conditions, *F. nucleatum* can be more resistant to oxidative stress and host response [6]. As shown in Table 1, *xanthorrhizol* has an antibacterial effect against the biofilm of *F. nucleatum*. This study is similar to that of Kim (2008), which showed that *xanthorrhizol* has an antibacterial effect against *S. mutans* biofilms. The *post hoc* Mann–Whitney U test was used to assess these differences.

As shown in Table 2, there were significant differences in the eradication value against the biofilm of *F. nucleatum* between *xanthorrhizol* at concentrations of 1.25%, 0.5%, 0.75%, and 1%. However, there was no significant difference between *xanthorrhizol* at a concentration of 1.5% and 2.5% NaOCl. Moreover, the eradication value between *xanthorrhizol* at all concentrations, except 0.5%, and 2.5% NaOCl, did not differ significantly.

On this study, the optimal *xanthorrhizol* concentration was 1.25% (Table 2). Meanwhile, Kim's study (2008), the optimal concentration was 1%. The difference in optimal concentration is attributed to the different bacteria species that were tested [19] Yue *et al.* (2015) showed that *xanthorrhizol* has an antibacterial effect against *E. faecalis* ATCC 29212 biofilm. The optimal concentration in this study was different because a concentration of 1% on Yue's study (2015) was used for *xanthorrhizol* and it was dissolved with 30% ethanol, 1% DMSO, and 100 mg/mL of sodium methyl cocoyl taurate [24].

Table 3 represents the total count of *F. nucleatum* biofilms on the well plate. There was a significant difference in the total count of *F. nucleatum* biofilms after exposure to *xanthorrhizol* at concentrations of 0.5%, 0.75%, 1%, 1.25%, and 1.5% and 2.5% NaOCl.

Significant differences were observed in the *F. nucleatum* biofilm colony count between *xanthorrhizol* at various concentrations and 2.5% NaOCl. As shown in Table 4, the *post hoc* Mann–Whitney U test was used to assess the difference in colony count between the various concentrations of *xanthorrhizol*.

There was a significant difference in the number of colony count between *xanthorrhizol* at a concentration of 1% and at 1.25% and 1.5% and 2.5% NaOCl. Meanwhile, there were no significant differences between *xanthorrhizol* at concentrations of 1.25% and 1.5% and 2.5% NaOCl.

This study, similar to Prijatmoko's study (2018), has shown that the active compound in essential oils made from turmeric (*Curcuma xanthorrhiza* Roxb.) can inhibit *F. nucleatum* growth [21].

F. nucleatum has been used as ATCC 25586, which is one of *F. nucleatum*'s characteristics in the root canal [1]. Future studies must use samples of clinical isolates to identify the actual strain of microorganisms in the root canal.

NaOCl is an irrigation solution that is most used in endodontic treatments. Al-Madi *et al.* (2019) have shown that 2.5% NaOCl has antibacterial effects against *F. nucleatum*. This solution has an

Table 1: Eradication value (%) between *xanthorrhizol* at various concentrations and 2.5% NaOCl against the biofilms of *F. nucleatum* ATCC 25586

Treatment group (concentrations)	n	Median (min–max) (%)	95% CI		p value
			Lower limit	Upper limit	
Xanthorrhizol (0.5%)	6	70.0 (13.2–76.9)	33.3	84.5	0.006*
Xanthorrhizol (0.75%)	6	77.0 (63.7–87.2)	68.8	85.3	
Xanthorrhizol (1%)	6	71.6 (64.1–82.6)	64.8	79.5	
Xanthorrhizol (1.25%)	6	86.8 (81.3–91.3)	82.8	90.7	
Xanthorrhizol (1.5%)	6	83.5 (71.8–87.1)	74.2	88.4	
2.5% NaOCl	6	86.4 (0–0.103)	56.0	90.2	

*Kruskal–Wallis test with a p<0.05

Table 2: Eradication value (%) against the biofilm of *F. nucleatum* between *xanthorrhizol* at various concentrations and 2.5% NaOCl

Testing materials (concentration)	Xanthorrhizol					
	0.5%	0.75%	1%	1.25%	1.5%	2.5% NaOCl
Xanthorrhizol (0.5%)		0.055	0.423	0.004*	0.025*	0.025*
Xanthorrhizol (0.75%)			0.297	0.025*	0.630	0.200
Xanthorrhizol (1%)				0.006*	0.054*	0.078
Xanthorrhizol (1.25%)					0.149	0.631
Xanthorrhizol (1.5%)						0.630
2.5% NaOCl						

**Post hoc* Mann–Whitney U test with a p<0.05

Table 3: *F. nucleatum* biofilm colony count (CFU/mL) after exposure to *xanthorrhizol* at various concentrations and 2.5% NaOCl

Treatment group (concentration)	n	Median (min–max) (CFU/mL)	95% CI		p value
			Lower limit	Upper limit	
Xanthorrhizol (0.5%)	6	1.00 (0–11)	–1.50	8.50	0.008*
Xanthorrhizol (0.75%)	6	5.00 (0–18)	–1.59	16.59	
Xanthorrhizol (1%)	6	4.00 (2–9)	2.32	8.02	
Xanthorrhizol (1.25%)	6	0.00 (0–1)	–0.26	0.60	
Xanthorrhizol (1.5%)	6	0.00 (0–1)	–0.26	0.60	
2.5% NaOCl	6	0.00 (0–1)	–0.26	0.60	

*Kruskal–Wallis test with a p<0.05

Table 4: Total count of the biofilm of *F. nucleatum* colonies on the well plate after exposure to various concentrations of xanthorrhizol and 2.5% NaOCl

Materials (concentration)	Xanthorrhizol					
	0.5%	0.75%	1%	1.25%	1.5%	2.5% NaOCl
Xanthorrhizol (0.5%)		0.739	0.224	0.072	0.072	0.072
Xanthorrhizol (0.75%)			1.000	0.153	0.153	0.153
Xanthorrhizol (1%)				0.003*	0.003*	0.003*
Xanthorrhizol (1.25%)					1.000	1.000
Xanthorrhizol (1.5%)						1.000
2.5% NaOCl						1.000

*Post hoc Mann-Whitney U test with a p<0.05

unpleasant smell and taste, and it may trigger allergic reactions. Moreover, it causes fibroblast cytotoxicity at concentrations greater than 0.05% [14].

Curcuma xanthorrhiza Roxb., also known as temulawak or Javanese turmeric, is widely used in medicines [25]. Xanthorrhizol, an active compound in essential oils obtained from the fractionation of *Curcuma xanthorrhiza* Roxb. plant, has antibacterial, antifungal, anticancer, antioxidant, and anti-inflammatory properties. In this study, xanthorrhizol, derived from the fractionation of ginger (*Curcuma Xanthorrhiza* Roxb.) obtained from PT Tri Rahardja (Javaplant), Surakarta, was used. The separation of active substances using the fractionation method aims to obtain more optimal antibacterial effects [26]. In addition, fractionation compounds have a more potent activity than the extract form, and fractionation techniques can optimally eliminate or separate unwanted compounds [27].

Xanthorrhizol's antibacterial effect was attributed to phenol and hydrocarbon chain mechanisms. Phenolic compounds consist of hydroxyl, which interacts with bacterial cells through an adsorption process. In this process, hydrogen bonds may change the bacterial cell membrane permeability. Phenol's penetration of the bacterial cells can cause coagulation of proteins that will lyse the bacterial cell membranes [25].

CONCLUSION

Xanthorrhizol at various concentrations (0.5%, 0.75%, 1%, 1.25%, and 1.5%) has antibacterial effects against *F. nucleatum* biofilms. Moreover, the effect of xanthorrhizol at concentrations of 1.25% and 1.5% is similar to that of 2.5% NaOCl, which is effective in eliminating *F. nucleatum* biofilm.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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