COMPARISON OF ANTIFUNGAL EFFECT OF XANTHORRHIZOL (CURCUMA XANTHORRHIZA ROXB.) AND 2% CHLORHEXIDINE AGAINST CANDIDA ALBICANS AMERICAN TYPE CULTURE COLLECTION 10231 BIOFILM

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INTRODUCTION

Endodontic infection is not only caused by the presence of bacteria but also by fungi. The invasion of fungi can be found in primary, secondary, and persistent endodontic infections [1,2]. Siquerra et al. and Shah et al. reported that fungi have rarely been found in primary endodontic infections (prevalence: 8%), but they seem to occur more often in persistent endodontic infections (prevalence >18%) [1-3]. Several species of fungi have been detected in the root canal system including, Candida, Aspergillus, Penicillium, and Fusarium species [4]. From among these species, Candida albicans is the most commonly observed species found in persistent endodontic infections [3,5]. Biofilm formation is one of the important virulence attributes of C. albicans because C. albicans cells growing in a biofilm can be 1000-fold resistant to antifungal agents that are planktonic cells [6-8]. Several studies have shown that 2% chlorhexidine (CHX) is an effective irrigation solution against C. albicans [9,10]. Although 2% CHX has been claimed to be a safe irrigation solution in endodontic treatment, Chang et al. found that CHX with a concentration of more than equal to 0.0001% was toxic to periodontal ligament cells [11]. Liu et al. also observed that 2% CHX permanently halts cell migration and significantly reduced the survival of fibroblasts and osteoblasts [12]. These toxic effects can cause degeneration of the periapical tissue and delay healing [11]. Thus, further research is necessary to elucidate an effective irrigation solution against C. albicans biofilm without imparting toxic effects to host cells [13].

Curcuma xanthorrhiza Roxb., commonly known as Java turmeric, has been used as traditional medicinal plant in Indonesia for food and medicinal purposes. C. xanthorrhiza Roxb. contains xanthorrhizol (XNT) and curcuminoid as its active compounds [14]. XNT is the most active compound that can only be isolated from the essential oil of the rhizomes of C. xanthorrhiza Roxb. [15]. Helen et al. reported that essential oil extracted from the rhizome of C. xanthorrhiza Roxb. was effective in inhibiting the growth of several fungi, including C. albicans. The dominant XNT content in essential oils used in their research indicated that XNT has an antifungal effect [13]. Meanwhile, Rukayadi et al. found that 32 µg/mL of XNT was able to eradicate 67.48% of C. albicans biofilm [16].

In this study, the antifungal effect of XNT against C. albicans American Type and Culture Collection (ATCC) 10231 biofilm was analyzed at several concentrations so that the optimum concentration of XNT against C. albicans biofilm might be found and its effects could be compared with those of 2% CHX solution.

METHODS

Culture of C. albicans

C. albicans ATCC 10231 (obtained from the Oral Biology Laboratory cultured stock) was cultured aerobically on Sabouraud Dextrose Agar (SDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C for 48 h. Following the incubation time period, C. albicans was collected with an ose needle until one full loop was obtained, and then inserted into a tube containing 10 mL of Sabouraud Dextrose Broth (SDB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) solution. The tube was centrifuged using a vortex mixer, then incubated aerobically at 37°C for 48 h. Following the incubation time period, C. albicans was cultured aerobically on Sabouraud Dextrose Agar (SDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) solution. The tube was centrifuged using a vortex mixer, then incubated aerobically in an incubator at 37°C for 48 h and stored at 4°C in the refrigerator before use. Then, C. albicans suspension was diluted to concentrations of 10⁻⁰, 10⁻¹, and 10⁻². Three Eppendorf tubes labeled according to the concentration were prepared in sequence and filled with 990 µL of SDB. Next, 10 µL of C. albicans suspension was added into the Eppendorf tube labeled as 10⁻⁰ to create a 10⁻² suspension and then homogenized with a vortex mixer. A 10⁻⁰ suspension was made by adding 10 µL of 10⁻² C. albicans suspension into an Eppendorf tube labeled 10⁻⁰ and
homogenizing it. The same procedure continued until a 10^-6 C. albicans suspension was obtained. Then, C. albicans suspension from each concentration was cultured on SDA at 37°C for 48 h. According to the number of C. albicans colonies that could be counted visually, the 10^-6 concentration of C. albicans suspension was used further in this study.

**C. albicans biofilms formation**

The formation of C. albicans biofilm was conducted by adding 100 µL of 10^-6 (containing 1 x 10^8 cells/mL) of C. albicans suspension into flat-bottom 96-well microtiter plates (IWAKI; Asahi Glass Co., Ltd., Japan), according to the well design. Then, 100 µL of SDB was added into each well, as growth medium, followed by 48 h of incubation at 37°C to facilitate cell attachment and biofilm formation. After incubation, the medium was discarded and non-adherent cells were removed by washing the biofilm with sterile phosphate-buffered saline (PBS) (Oxoid; Thermo Fisher Scientific, Waltham, MA, USA). At this point, C. albicans biofilm samples were ready to be exposed to herbal testing.

**Preparation of antifungal agent**

The XNT (obtained from PT. Tri Rahantia; Javapiant, Indonesia) was isolated from the rhizome of C. xanthorrhiza Roxb. XNT has a brown color with a thick consistency. The analysis of XNT content was performed using gas chromatography–mass spectrometry at the Laboratory Kesehatan Daerah in Labkesda, Indonesia, which revealed that the XNT used in this study had a concentration of 95%. XNT was dissolved in dimethyl sulfoxide (EMSURE; Merck, Darmstadt, Germany) to obtain 0.25% XNT, 0.5% XNT, 0.75% XNT, 1% XNT, and 1.25% XNT solutions.

**3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyterazolium bromide (MTT) assay**

C. albicans biofilm in microplates was exposed to 100 µL of XNT with different concentrations (0.25%, 0.5%, 0.75%, 1%, and 1.25%) to be tested. In conjunction, 2% CHX (100 µL) was added into the positive control well, while 100 µL of SDB was added into the negative control well (i.e., biofilm without added material for testing). In this study, the XNT solutions of varying concentrations were also included as blank samples. Next, the well plates were incubated for 15 min at 37°C and then washed once with 100 µL of PBS solution.

The metabolic activity of C. albicans biofilm was assessed using MTT reduction assay (Sigma-Aldrich, St. Louis, MO, USA). The MTT salt was dissolved in PBS to give a final concentration of 5 mg/mL. The MTT solution was kept in 15 mL tubes and was wrapped with aluminum foil until used. Next, 200 µL of MTT solution (5 mg/mL) was added to each well and wrapped in aluminum foil before incubating for 3 h at 37°C aerobically. After incubation, 200 µL of acidified isopropanol solution was added to each well to stop the reaction. The plates were shaken by an orbital shaker (80 rpm) at room temperature for 1 h. Finally, the solution’s optical density (OD) was measured by a microplate reader at a wavelength of 570 nm. The percentage of biofilm eradication was calculated by the following formula [17,19]:

\[
\text{Percentage of biofilm eradication(%) = } \left(1 - \frac{\text{OD sample} - \text{OD blank sample}}{\text{OD negative control} - \text{OD blank negative control}}\right) \times 100%
\]

The OD sample is the OD of C. albicans biofilm after exposure to material tested, the OD blank sample is the OD of material tested; the OD negative control is the OD of C. albicans biofilm after exposure to SDB (negative control); and the OD blank negative control is the OD of SDB (blank negative control).

**Total plate count (TPC)**

C. albicans biofilm in microplates was exposed to 100 µL of XNT with several concentrations (0.25%, 0.5%, 0.75%, 1%, and 1.25%). The negative control was the C. albicans biofilm unexposed to any antifungal agent and the positive control was the C. albicans biofilm exposed to 2% CHX. Next, the well plates were incubated for 15 min at 37°C and then washed once with 100 µL of PBS solution. Antifungal testing using the TPC method was done by scraping the bottom of each well (after adding 100 µL of PBS) with an Eppendorf tip and 10 µL suspension was taken to be cultured on SDA medium aerobically at 37°C for 48 h. Following incubation, the number of colonies was visually counted and data were recorded.

**Statistical analysis**

Data were analyzed using one-way analysis of variance with significance set at p<0.05. We used the Statistical Package for the Social Sciences version 22.0 software program (IBM Corp., Armonk, NY, USA).

**RESULTS AND DISCUSSION**

In this study, XNT isolated from the rhizome of C. xanthorrhiza Roxb. was tested against C. albicans biofilm. C. albicans is by far the fungal species most commonly isolated from infected root canal with periduricular disease due to the failure of root canal treatment [1]. C. albicans is eukaryotic microorganisms that can take part in endodontic infections and thereby may participate in the etiology and pathogenesis of periduricular disease. They possess virulence attributes, including adaptability to a variety of environmental conditions, adhesion to a variety of surfaces, the production of hydrolytic enzymes, morphologic transition, and evasion of the host defense. Moreover, C. albicans biofilm is 1000 fold resistant to host defenses and antifungal agents, leading to more difficulty in treating persistent endodontic infection due to the presence of C. albicans [7,8,19]. Therefore, in this study, antifungal testing was performed on C. albicans biofilm with the aim of that the results can be useful to increase the success rate in endodontic treatment, especially of persistent endodontic infection.

Recently, interest in natural antifungal compounds has been increasing. Natural compounds have been proven to have better compatibility with biological systems and less side effects [14]. Some essential oils have been shown to possess antifungal properties. However, the specific active agent in the essential oils has not been identified and it is possible that minor components of the essential oils may exhibit toxic or adverse effects in vivo. XNT, an essential oil compound derived from the rhizome of Java turmeric (C. xanthorrhiza Roxb.), could be a strong candidate for eliminating C. albicans biofilm [6]. XNT is a bisaboline-type sequestration compound proven to possess antifungal activity.

The C. albicans biofilm ATCC 10231 reference strain was tested in this study because this strain has been reported as commonly found in the oral cavity. Incubation at 37°C can support C. albicans growth and formation of pathogenic hyphae. A 48 h incubation period was chosen in this research because it is the optimum time for C. albicans to form biofilm [20].

In our research, various concentrations of XNT were exposed to C. albicans biofilm for 15 min, referring to the previous research conducted by Sera et al. In their study, 2% CHX solution eliminated all biofilms of various microorganisms within 15 min, one of which was C. albicans biofilm [21]. This research is also in accordance with that conducted by Shubham et al., who found that papaya extract solution had an effective antifungal effect on C. albicans after 15 min of exposure [22].

In this study, antifungal test results for C. albicans biofilm were observed by MTT assay and TPC. The antifungal effectiveness of various concentrations of XNT solution were compared with that of 2% CHX solution as a positive control. The absorbance value (OD) of the MTT assay test was converted into a formula to calculate the percentage of biofilm eradication.

Table 1 shows the percentage of biofilm eradication of XNT at different concentrations and 2% CHX against C. albicans biofilm. Based on the median values observed, all XNT solutions used in this study achieved a percentage of eradication, with values ranging from 30% to 99%. Thus,
all XNT solutions used in this study have antifungal effects on *C. albicans* biofilm.

The difference in significance in each group was tested using the Kruskal-Wallis test, following a normality test with Shapiro–Wilk, which showed non-normal data. The Kruskal-Wallis test results revealed p=0.002 (p<0.05). These results indicate that at least there were differences in eradication value between the two groups of six concentrations tested.

Furthermore, to elucidate the groups with different eradication abilities, a post hoc analysis with the Mann–Whitney U-test was conducted. The significance values (p-values) from the results of post hoc analysis are summarized in Table 2.

Based on the statistical analysis in Table 2, it was found that all XNT solutions had the ability to inhibit *C. albicans* biofilm to a degree that differed depending on the concentration used. In this study, it was revealed that 1% XNT solution was able to reduce 90% or more of the viability of *C. albicans*. Meanwhile, another study found that *C. xanthorrhiza* extract at a concentration of 45% could only eradicate approximately 60% of *C. albicans* biofilm [23]. This is assumed to be because the *C. xanthorrhiza* extract contained other compounds besides XNT, limiting the antifungal effect on *C. albicans* biofilm. The previous research by Rukayadi et al. found that 32 μg/mL was able to eradicate 67.48% of *C. albicans* biofilm. This is presumably because the dilution used in that study was too small so the optimum concentration was not obtained in eradicating *C. albicans* biofilm [16].

Antifungal test then continued with calculating the number of colonies (TPC method) after exposure to various concentrations of XNT solution and 2% CHX against *C. albicans* biofilm.

The results of antifungal testing using the TPC method are shown in Table 3.

According to Table 3, there was a difference in significance among each group tested by the Kruskal-Wallis test, following a normality test with Shapiro–Wilk, which showed non-normal data. The Kruskal-Wallis test results showed p=0.001 (p<0.05). These results indicate that there were differences in total colony growth between the two groups of six concentrations tested. Post hoc analysis findings conducted with the Mann–Whitney U-test are summarized in Table 4.

Based on Table 4, it can be seen that there was no difference in the total colony growth after exposure to 2% CHX, 1% XNT, and 1.25% XNT, which indicates that 2% CHX, 1% XNT and 1.25% XNT have equivalent antifungal effects on *C. albicans* ATCC 10231 biofilm.

From this research, statistical analysis (Table 2) showed that there was no significant difference in the percentage of biofilm eradication among 1% XNT, 1.25% XNT, and 2% CHX solutions, where the average percentages of biofilm eradication were 96.25%, 94%, and 93%, respectively. This finding is also similar to the results of TPC. Statistical analysis of the TPC results listed in Table 4 showed that there was no significant difference between 1% XNT, 1.25% XNT, and 2% CHX against *C. albicans* biofilm.

### Table 1: The median and p-values of 2% CHX and XNT solutions in eradicating *C. albicans* biofilm

| Groups          | n | Median value of percentage of biofilm eradication (%) (min–max) | 95% confidence interval | p-value   |
|-----------------|---|---------------------------------------------------------------|------------------------|-----------|
|                 |   |                                                               | Lower bound            | Upper bound |          |
| 2% CHX          | 4 | 92.5 (90–97)                                                  | 87.96                  | 98.03      | 0.002    |
| 0.25% XNT       | 4 | 40 (30–50)                                                    | 27                     | 52.99      |          |
| 0.5% XNT        | 4 | 45 (40–50)                                                    | 35.81                  | 54.18      |          |
| 0.75% XNT       | 4 | 60 (40–60)                                                    | 39.08                  | 70.91      |          |
| 1% XNT          | 4 | 96 (94–99)                                                    | 92.72                  | 96.22      |          |
| 1.25% XNT       | 4 | 94.5 (90–97)                                                  | 89.31                  | 98.68      |          |

Kruskal–Wallis test (p<0.05). CHX: Chlorhexidine, XNT: Xanthorrhizol, *C. albicans: Candida albicans*

### Table 2: Significance value (p-values) of differences in the ability of 2% CHX and XNT solutions in eradicating *C. albicans* biofilm

| Materials testing | 0.25% XNT | 0.5% XNT | 0.75% XNT | 1% XNT | 1.25% XNT |
|-------------------|-----------|----------|-----------|--------|-----------|
| 2% CHX            | 0.02*     | 0.019*   | 0.018*    | 0.144  | 0.659     |
| 0.25% XNT         |           | 0.343    | 0.069     | 0.02*  | 0.02*     |
| 0.5% XNT          |           |          | 0.127     | 0.019* | 0.019*    |
| 0.75% XNT         |           |          |          | 0.018* | 0.018*    |
| 1% XNT            |           |          |          |        | 0.304     |

Kruskal–Wallis test (p<0.05). *Indicates the presence of differences in the percentage of *C. albicans* ATCC 10231 biofilm eradication between groups. CHX: Chlorhexidine, XNT: Xanthorrhizol, *C. albicans: Candida albicans*

### Table 3: Median and p-values of total colony growth after exposure of 2% CHX and various concentrations of XNT against *C. albicans* biofilm

| Groups          | n | Median value of total colony (Min–Max) | 95% confidence interval | p-value   |
|-----------------|---|---------------------------------------|------------------------|-----------|
|                 |   |                                       | Lower bound            | Upper bound |          |
| 2% CHX          | 4 | 0                                     | Constant               | Constant   | 0.001    |
| 0.25% XNT       | 4 | 6 (5–6)                               | 4.95                   | 6.54       |          |
| 0.5% XNT        | 4 | 2 (2–3)                               | 1.45                   | 3.04       |          |
| 0.75% XNT       | 4 | 1 (0–2)                               | -0.29                  | 2.29       |          |
| 1% XNT          | 4 | 0                                     | Constant               | Constant   |          |
| 1.25% XNT       | 4 | 0                                     | Constant               | Constant   |          |

Kruskal–Wallis test (p<0.05). CHX: Chlorhexidine, XNT: Xanthorrhizol, *C. albicans: Candida albicans*
and 2% CHX, while no colony growth of *C. albicans* was observed following culture on SDA medium. Thus, it can be concluded that the antifungal effects of 1% XNT and 1.25% XNT were equivalent to that of 2% CHX. Therefore, according to the two antifungal tests we conducted, the optimal concentration of XNT against *C. albicans* biofilm was 1%.

XNT is a phenol derivative compound so it is assumed to have the same antifungal mechanism as other phenol derivatives. Phenol compounds have a high penetration rate into the cell membrane through hydrogen binding between hydroxyl groups in the phenol compound and cell membrane proteins, which cause a membrane permeability imbalance. This mechanism makes the essential components (i.e., protein, DNA, and RNA) of the cell leak out and cause cell death [15,24]. This is similar to the research conducted by Rukayadi et al., who found that XNT affected the external morphology, exhibiting deformation and protrusion of the cell surfaces of *C. albicans* in examination by scanning electron microscopy [25]. Rukayadi and Hwang (2006) also examined the mechanism of action of XNT against *Streptococcus mutans* biofilm. From their research, it was reported that XNT was able to reduce the viability of *Streptococcus mutans* biofilm because XNT could kill *Streptococcus mutans* cells in the outer layer of the biofilm as these cells have direct contact with XNT so that the dead cells could be degraded and released from biofilm, resulting in reduced biofilm viability [26]. Henriques et al. in their study stated that the *C. albicans* biofilm structure resembles the biofilm structure of oral bacteria species. Thus, a similar antifungal mechanism is assumed to occur with XNT against *C. albicans* biofilm [27].

**CONCLUSION**

In this study, XNT was proven to have an antifungal effect on *C. albicans* ATCC 10231 biofilm. We assumed that this natural herbal may be a potential candidate for use as an irrigation solution in endodontic treatment, especially in persistent endodontic infections where *C. albicans* is commonly detected in addition to other bacterial species (reference). In particular, the 1% XNT and 1.25% XNT solutions in this study had antifungal effects equivalent to that of 2% CHX solution against *C. albicans* ATCC 10231 biofilm. Thus, the optimum concentration of XNT solution to use against *C. albicans* ATCC 10231 biofilm is 1%. The results obtained herein can be further studied; aiming to evaluate the antifungal effect on XNT against *C. albicans* biofilm isolated from infected root canals so that the research of XNT as an irrigating solution in endodontic treatment can be further progressed.

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**CONFLICTS OF INTEREST**

The authors have declared that there are no conflicts of interest.

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21. Sena NT, Gomes BP, Vianna ME, Berger VB, Zaia AA, Ferraz CC, et al. Table 4: Significance values (p-values) of total colony growth after exposure of 2% CHX and various concentrations of XNT against *C. albicans* biofilm

| Material testing | 0.25% XNT | 0.5% XNT | 0.75% XNT | 1% XNT | 1.25% XNT |
|------------------|-----------|-----------|-----------|--------|----------|
| 2% CHX           | 0.011*    | 0.011*    | 0.046*    | 1      | 1        |
| 0.25% XNT        | 0.015*    | 0.017*    | 0.011*    | 0.011* | 1        |
| 0.5% XNT         | 0.011*    | 0.044*    | 0.011*    | 0.046* | 0.046*   |
| 0.75% XNT        |           |           |           |        |          |
| 1% XNT           |           |           |           |        | 1        |

Mann–Whitney U-test (p<0.05). *Indicates the presence of differences in the percentage of *C. albicans* ATCC 10231 biofilm eradication between groups.

CHX: Chlorhexidine, XNT: Xanthorrhizol, *C. albicans*: Candida albicans.

**Table 4: Significance values (p-values) of total colony growth after exposure of 2% CHX and various concentrations of XNT against *C. albicans* biofilm**
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