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The antibacterial effect of xanthorrhizol as an endodontic irrigant on Enterococcus faecalis

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ABSTRACT

The antibacterial effect of xanthorrhizol as an endodontic irrigant on Enterococcus faecalis

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Objectives The aim of this study was to evaluate the antibacterial effect of xanthorrhizol (XTZ) on *E. faecalis*, compared with 2% chlorhexidine (CHX).

Materials and Methods Normal physiological state (NS), starvation state (SS), and alkalization state (AS) of E. faecalis were used. A solution containing 1% XTZ in 30% ethanol, 1% dimethyl sulfoxide (DMSO), and 100 mg/ml sodium methyl cocoyl taurate was used and is referred to as Xan in this study. To determine the minimal bactericidal concentration (MBC) of Xan and CHX, 500 μl of *E. faecalis* (NS and two stress states) was added to a microtube containing 500 μl of serial 2-fold dilutions of 1% Xan and 2% CHX (1:2-1:128). The MBC of each antimicrobial was determined by the plate count method.

Results The antibacterial effect of Xan was more effective on *E. faecalis* in AS than in the other states (NS, SS) at 0.125% Xan and 0.03325% Xan ($P<0.05$). In contrast, the antibacterial effect of CHX was more effective against *E. faecalis* in SS than the other states (NS, AS) at 0.0625% CHX ($P<0.05$). In SS, the antibacterial effect of CHX was more effective than that of Xan at 0.125% and 0.0625% ($P<0.05$). However, in AS, the antibacterial effect of Xan was more effective than that of CHX at 0.0625% and 0.03325% ($P<0.05$).

Conclusions In endodontic retreatment cases in which it is important to effectively remove *E. faecalis* from the infected root canal, Xan may be more suitable when combined with NaOCl than CHX.

Key words : Xanthorrhizol · chlorhexidine · root canal irrigation · *E. faecalis*

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Ⅰ. Introduction

Bacteria have been recognized as the primary etiology in the development of periapical bone lesions[1], and endodontic treatment is a procedure to prevent or cure apical periodontitis caused by an infection of the root canal systems of affected teeth[2]. When endodontic treatment is performed under aseptic conditions and clinical principles, it is a predictable and reliable treatment with high success rates ranging from 86 to 98%[3]. Despite optimal endodontic therapy, endodontic failures still occur because there are root canal areas that cannot adequately be debrided with instrument and disinfected with chemical agents, and further retreatment is needed[4].

The need for retreatment is due either to reinfection by oral bacteria or, more often, to microorganisms persisting in the apical part of the root canals of filled teeth[5]. Enterococcus faecalis(*E. faecalis*) is known as the dominant microorganism in persistent apical periodon titis[6]. *E. faecalis*, a pathogenic microorganism, has various resistances to different environment; such as an alkaline and acidic environment, bile salt, starvation, and many antibacterial agents[7~9].

Sodium hypochlorite(NaOCl) is the most commonly used root canal irrigant[10]. It is an antiseptic and inexpensive lubricant that has various advantages such as ease of use, strong and fast oxidizing ability, broad spectrum antimicrobial effects and the ability to dissolve organic substances by breaking proteins down into amino acids. In spite of these advantages, it has also several drawbacks such as unpleasant odor and taste, cytotoxicity when injected into periradicular tissues, and, most of all, it does not kill all bacteria including *E. faecalis*[10, 11].

Various chemical antibiotic agents have been suggested as new endodontic irrigants, including chlorhexidine(CHX), a potent antimicrobial agent that is particularly effective against *E. faecalis*[12]. CHX also has various advantages, in addition to its biocompatibility and efficacy, this irrigant has a long-term antibacterial effect due to its adherence to hydroxyapatite[13]. However, CHX is not used as a routine irrigant, but rather for the final rinsing of the canal due to its inability to dissolve necrotic pulp tissue remnants[14]. Additionally, several studies reported that 2% CHX produced an orange-brown precipitate when associated with NaOCl solutions[15~17].

The Curcuma xanthorrhiza extract was isolated from the ethyl-acetate fraction of the methanol extract of Javanese turmeric(Curcuma xanthor rhiza Roxb.), a medicinal agent in Indonesia. Xanthorrhizol(Fig. 1, XTZ), which is the main active component of the xanthorrhiza extract[18], has various pharmacological characteristic, such as anti-metastasis, inhibitory effects on nephrotoxicity, anti-cancer and anti-inflam matory effects[19~23]. Previous studies reported that XTZ has an antibacterial effect on mutans streptococci and reducing gingivitis[24, 25]. However, to the best of our knowledge, there is no study about antibacterial effect of XTZ against *E. faecalis*. Therefore, the aim of this study was to evaluate the antibacterial effect of XTZ as a new endodontic irrigant against *E.*

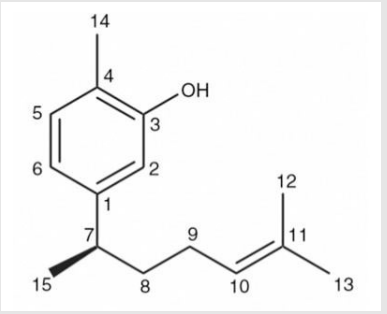

Fig. 1. Structure of xanthorrhizol.

faecalis, in comparison with 2% CHX.

Ⅱ. Materials & Methods

Bacteria strains and culture conditions

E. faecalis(ATCC 29212; BOSUNG SCIENTIFIC Co., KOREA) was used in the present study. The bacteria were streaked from the frozen stock culture onto 25 ml brain heart infusion (BHI; Difco Co., Becton Dickinson, Sparks, MD, USA) in a conical tube at 37℃ for 24 hours under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) in an incubator(FORMA1029; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Test Compounds

The Curcuma xanthorrhiza extract was obtained from the Bioproducts Research Center of Yonsei University and isolated from the ethyl acetate fraction of the methanol extract of Curcuma xanthorrhiza Roxb. using the method of Hwang et al.[18, 26], silica gel column chromatography(Merck; 70-230 mesh; 5 x 43 cm; n-hexane/ethyl acetate, 10:1). However, XTZ is so fat-soluble that it has to be solubilized in order to be used as an endodontic irrigant. Based on our pilot study for the solubilization of XTZ, 1% XTZ in 30% ethanol, 1% dimethyl sulfoxide (DMSO), and 100 mg/ml sodium methyl cocoyl taurate was used and is referred to as Xan in this study. Two percent CHX(Sigma-Aldrich Co., St. Louis, MO, USA) and mixed solvent without XTZ; 30% ethanol, 1% DMSO, and 100mg/ml sodium methyl cocoyl taurate were used as the control.

Bacterial Preparation

In this study, the *E. faecalis* starvation(SS) and pretreatment alkalization(AS) states based on the methods of Tong et al[27]. were considered stress states and were used in addition to normal physiological state(NS). Briefly, to prepare *E. faecalis* in the SS, *E. faecalis* was centrifuged by 6000 rpm at 4℃ for 5 minutes, and the supernatant was discarded. The cell deposit was washed with sterile PBS(phosphate buffered saline;

Lonza Inc., Allendale, NJ, USA) twice, resuspen ded in PBS and stored at 37℃ for 2 days. The starved cells were 10-fold diluted for a plate count to quantify the viable bacterial cell concentration. To prepare *E. faecalis* in the AS, the above cell deposit was pretreated in a solution of calcium hydroxide power and distilled water($Ca(OH)_2$, pH 10.3) for 2 hours, and the supernatant was then discarded after centrifugation. The cell deposit was resuspended in PBS. The viable bacterial cell concentration was evaluated by the plate count method. The quantitative determination was confirmed on every experimental days.

To determine the antibacterial effect of Xan and CHX on *E. faecalis*, *E. faecalis* in the NS was adjusted to approximately 3 x 10^9 CFU/ml and 4 x 10^8 CFU/ml in the 2 stress states (SS, AS) with BHI broth according to the above quantitative determination of cells in each conditions.

Antibacterial Assay

The minimal bactericidal concentration(MBC) of Xan and CHX was determined by the plate count method. The MBC is defined as the lowest concentration of an antimicrobial required to kill a particular bacteria. For the MBC assay, 500 ㎕ of *E. faecalis*(normal physiological state and 2 stress states) was added to a microtube containing 500 ㎕ of serial 2-fold dilutions of 1% Xan and 2% CHX (1:2-1:128) and mixed solvent of 30% ethanol, 1% DMSO, and 100mg/ml sodium methyl cocoyl taurate. After mixing with a vortex mixer (Analog Vortex Mixer, Fisher Scientific™, Canada), 100 ㎕ was streaked on BHI agar plates and incubated at 37℃ for 24 hours under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2). Afterward, the MBC of each antimicrobial was determined by the plate count method

Statistical Analysis

SPSS 21.0(SPSS Inc, Chicago, IL, USA) was used for statistical analysis. To determine the antibacterial effect of CHX and Xan on each of three states of *E. faecalis*, the Kruskal-Wallis test was used. To Compare the antibacterial effect of Xan and CHX at the same concentration, in each of the three different state of *E. faecalis*, the Mann-Whitney U test was used. In all analyses, CFU/ml of *E. faecalis* was transformed using $\log_{10}$ and the level of significance was set at 0.05.

Ⅲ. Results

The MBC of Xan and CHX was the same, 0.25%, except for AS treated with Xan(Fig. 2, 3). The MBC of Xan in the AS was 0.125% (Fig. 2). Mixed solvent without XTZ; 30% ethanol, 1% DMSO, and 100mg/ml sodium methyl cocoyl taurate had no antibacterial effect on *E. faecalis* (Fig. 4).

The antibacterial effect of Xan was greater against *E. faecalis* in AS than the other states (NS, SS) at 0.125% Xan and 0.03325% Xan, respectively ($P<0.05$) (Fig. 2). In contrast, the

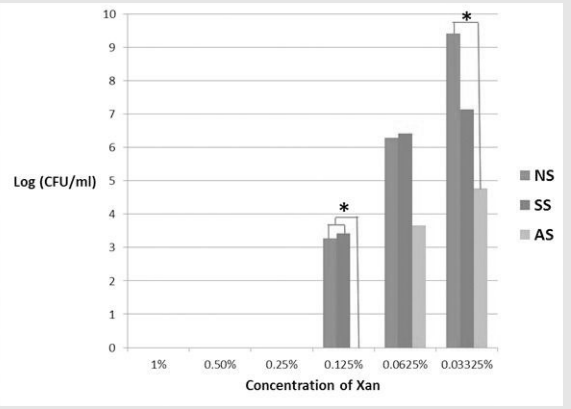

Fig. 2. The MBC of Xan in the experimental groups and the results of the Kruskal-Wallis test. MBC, Minimum bactericidal concentration; Xan, 1% Xanthorrhizol in 30% ethanol, 1% dimethyl sulfoxide (DMSO), and 100 mg/ml sodium methyl cocoyl taurate; NS, Normal state of E. faecalis; SS, Strarvation state of E. faecalis; AS, Alkalization state of E. faecalis. * Statistically significant difference ($p<0.05$).

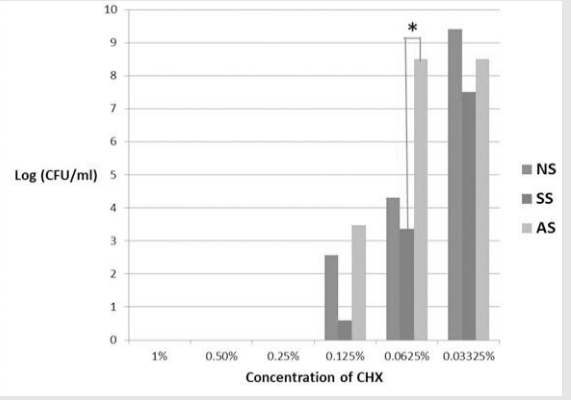

Fig. 3. The MBC of CHX in the experimental groups and the results of the Kruskal-Wallis test. MBC, Minimum bactericidal concentrationl; CHX, Chlorhexidine; NS, Normal state of E. faecalis; SS, Starvation state of E. faecalis; AS, Alkalization state of E. faecalis. * Statistically significant difference ($p<0.05$).

antibacterial effect of CHX was greater against *E. faecalis* in SS than the other states (NS, AS) at 0.0625% CHX (P<0.05) (Fig. 3).

In SS CHX was a more effective antibacterial agent than Xan at 0.125% and 0.0625% (P<0.05) (Fig. 5). However, in AS, the antibacterial effect of Xan was greater than that of CHX at 0.0625% and 0.03325% (P<0.05) (Fig. 6).

Ⅳ. Discussion

The goal of root canal treatment is to prevent or cure apical periodontitis by removing or at least reducing bacteria in the infected root canal[2]. Biomechanical cleaning and shaping of the root canal greatly reduces the number of bacteria, but because of the complexity of the canal anatomy, mechanical instrumentation cannot eliminate all

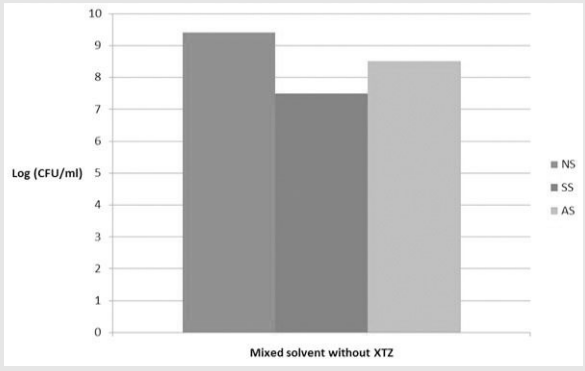

Fig. 4. Antibacterial effect of mixed solvent without XTZ; 30% ethanol, 1% DMSO, and 100mg/ml sodium methyl cocoyl taurate. NS, Normal state of E. faecalis; SS, Starvation state of E. faecalis; AS, Alkalization state of E. faecalis.

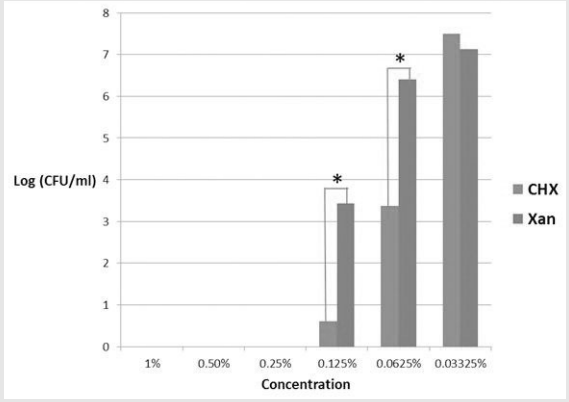

Fig. 5. Antibacterial effect in SS and Mann-Whitney U test results. CHX, Chlorhexidine; Xan, 1% Xanthorrhizol in 30% ethanol, 1% dimethyl sulfoxide (DMSO), and 100 mg/ml sodium methyl cocoyl taurate; SS, Starvation state of E. faecalis. * Statistically significant difference ($p<0.05$).

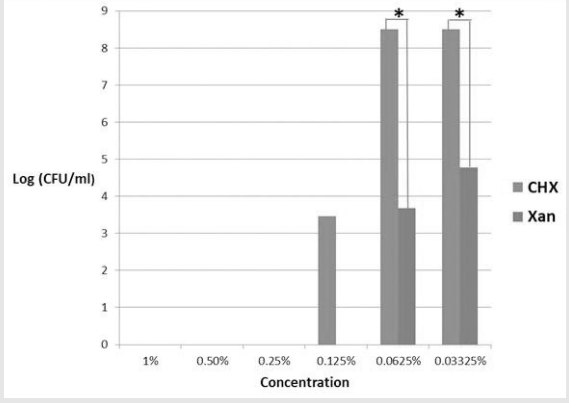

Fig. 6. Antibacterial effect in AS and Mann-Whitney U test results. CHX, Chlorhexidine; Xan, 1% Xanthorrhizol in 30% ethanol, 1% dimethyl sulfoxide (DMSO), and 100 mg/ml sodium methyl cocoyl taurate; AS, Alkalization state of E. faecalis. * Statistically significant difference ($p<0.05$).

infected tissue and bacteria present in isthmuses and ramifications[28]. Therefore, root canal irrigation with strong antibacterial agents in association with mechanical instrumentation is imperative to complete the cleaning and shaping process[29].

E. faecalis is associated with different forms of periradicular disease including primary endodontic infections and persistent infections. The frequency of *E. faecalis* found in persistent periradicular lesions has been shown to be quite high. In fact, failed root canal treatment cases are nine times more likely to contain *E. faecalis* than primary endodontic infections[30]. Therefore, E.faecalis is believed to play a major role in the etiology of failed root canals with persisting periradicular lesions[12]. Portenier et al.[31] states that it is probable that the physiological state of *E. faecalis* in the canal, particularly in retreatment cases, is closest to the starvation phase because bacterial cells in the root canal encounter a harsh ecological milieu. Meanwhile, $Ca(OH)_2$ has long been recommended clinically as an intracanal medicament between appoint ment due to its antibacterial effects, alkaline pH, and biocompatibility[13]. In the present study, three states of *E. faecalis* ; NS, SS, AS, were designed to evaluate the antibacterial effect of Xan and CHX in these clinical conditions.

The *E. faecalis* in NS in this study was used at approximately 3 x 10^9 CFU/ml and the amount used in the starvation and alkalization conditions was approximately one-tenth or 4 x 10^8 CFU/ml. It is well known that *E. faecalis* has acid-resistance as a result of the activity of the cell membrane-bound proton-translocating ATPase (H+-ATPase)[32]. Additionally, Kakinuma and Igarashi[33] proposed that in *E. faecalis*, an ATP-linked potassium/proton antiport system functions to bring protons into cells to combat intracellular alkalization. *E. faecalis* has high acid- and alkali-resistance because of the ATP-linked proton-transport systems that provides an additional means of maintaining pH homeostasis, so it was able to grow at pH 4.0-11.0[8]. Some studies reported that starvation has been shown to increase *E. faecalis* resistance to chemical, osmotic, and oxidative stress and 5.25% NaOCl[34, 35], because cells in starvation may slow their growth as the result of some nutrient limitation. Slow growth can account for biofilm resistance to antimicrobial agents[9]. Other studies stated that starvation triggers the synthesis of stress proteins, and these starvation-induced proteins can protect *E. faecalis* against attack[34, 36].

However, the results of the present study showed that the antibacterial effect of Xan and CHX in SS and AS was similar to or even greater that in NS. Tong et al.[27] also reported that *E. faecalis* in the starvation or alkalization states was more sensitive to MTAD, MTAN(substitu tion of doxycycline with nisin), and MTADN (nisin in combination with doxycycline) than in normal state. The authors stated that this result might be a result of the acidity of the 4.25% citric acid in MTAD. CHX is also acidic, and, the SS state of *E. faecalis* could not resist the acid attack because of nutritional deficiency. *E. faecalis* in the AS and SS states needs to generate energy, such as ATP, continuously because the cell

membrane-bound proton-transport systems requires ATP to function. Therefore, *E. faecalis* in stressed states might have difficulty responding immediately to the sudden presence of Xan and CHX.

CHX ($C_{22}H_{30}Cl_2N_{10}$) is a synthetic material comprising two biguanide groups and two symmetric 4-choloropheynyl rings connected by a hexa-methylene chain[37]. CHX acts by absorbing to the cell wall of the microorganism and causing leakage of cytoplasmic substances. Although CHX is a broad-spectrum antibacterial agent that is effective against Gram(+) and Gram(-) bacteria, it is less effective against Gram(-) microorganisms than against Gram(+) ones[38]. Additionally, because of its lack of tissue solubility, it is used with 2.5% NaOCl during instrumentation, and a final flush of canals is performed with 2% CHX[14]. However, the presence of NaOCl in the canals during irrigation with CHX can produce an orange-brown precipitate known as paracholoroaniline(PCA)[15, 16]. The formation of the precipitate could be explained by the acid-base reaction that occurs when NaOCl and CHX are mixed and PCA is the main by-product[15]. PCA, whose molecular formula is C_6H_6ClN, has been shown to be cytotoxic in rats[39] and the International Agency for Research on Cancer has listed it as group 2B "possibly carcinogen to humans"[40]. However, Prado et al.[17] reported that 2% CHX produced an orange-brown precipitate when associated with NaOCl solutions due to chlorination of the guanidino-nitrogens of CHX, but suggested that is not PCA. He suggested that the different results may be due to different detection techniques.

XTZ(Fig. 1), which is also known as 2-methyl-5-[(2r)-6-methylhept-5-en-2-yl]phenol; EINECS 250-090-2; (R)-5-(1,5-dimethyl-4-hexenyl)-o-cresol, consists of phenol and a hydrocarbon chain. Some studies have shown that XTZ has strong bactericidal activity against S. mutans biofilm[24, 41]. Although the precise antibacterial mechanism of XTZ is unclear, it might disturb or destroy the peptidoglycan layer of S. mutans[24]. The antibacterial activity of XTZ is significantly higher than that of carvacrol(5-Isopropyl-2-methylphenol), which is a commercial germicide with a similar chemical structure to XTZ, differing only in the length of the hydrocarbon chain[42]. It is therefore thought that the hydroxyl group is responsible for the main active antibacterial effect[24]. Additionally, XTZ is active against C. albicans biofilm so that XTZ has potential therapeutic implications against biofilm-associated candida infections[43].

In the present study, CHX displayed a greater antibiotic effect than Xan on *E. faecalis* in SS, while the opposite was true for *E. faecalis* in AS. The MBC for Xan against AS is 0.125%, lower than the 0.25% of CHX(Fig. 2). It is thought that there is a synergic effect between alkalization state of bacteria and the effect of Xan. Kakinuma and Igarashi[33] reported that an additional mechanism of membrane durability against acid and alkaline substances is involved in the acid- and alkali-resistance of *E. faecalis*, in addition to ATP-linked proton-transport systems function. Xan might disturb the peptidoglycan layer of *E.*

faecalis in the SS state, which could reduce its membrane durability. Therefore, according to these results, Xan appeared to be the more valuable endodontic irrigant in chronic periradicular lesions that require use of $Ca(OH)_2$ as an interappointment medicament.

There are some limitations of this experiment. Our study of the antibacterial effect of Xan was performed only in *E. faecalis*. Although previous studies reported that XTZ has an antibacterial effect on mutans streptococci and reducing gingivitis, its antibacterial effect on other bacteria found in the infected root canal, such as Enterococci and Streptococci, Lactobacilli, Peptostreptococci, Eubacterium alactolyticus, and Propionibacterium[44], needs to be determined in order for it to be used as an endodontic irrigant. Additionally, unlike intracanal dressing, which might remain in the root canal for 7 days or more, the contact time for antimicrobial agent in the intracanal irrigant is limited to the instrumentation time. In our study, BHI agar streaked with *E. faecalis* was treated with the antimicrobial at 37℃ for 24 hours under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2). Further study needs to be performed on the other bacteria using clinically base experimental conditions. However, because Xan has never been studied as an endodontic irrigant, this study presents the possibility of its use as a new endodontic irrigant.

In conclusion, the present study shows that, under the limited experimental conditions, Xan and CHX have a similar antibacterial effect on *E. faecalis*, but, especially in AS, Xan is more effective than CHX. Therefore, in endodontic retreatment cases in which it is important to effectively remove *E. faecalis* from the infected root canal, Xan may be a more suitable choice when combined with NaOCl than CHX.

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