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Antimicrobial effectiveness of *Chamaecyparis obtuse* extracts for intracanal bacteria removal using *Enterococcus faecalis*.

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Abstract

This study aimed to investigate the antimicrobial activity of *Chamaecyparis obtusa*extract on *Enterococcus faecalis (E. faecalis)*, typical causative bacteria that causes endodontic treatment failure and pulpitis. *Chamaecyparis obtusa* was immersed in 70% ethanol for 12 h, and concentrated *Chamaecyparis obtusa* extract was applied to *E. faecalis* diluted to 1×10^6 CFU/ml at the 0, 1.25, 2.5, 5, 10, 20, and 30 mg/ml concentrations. Then the colony-forming units (CFUs) were checked at 24 h to evaluate the antimicrobial activity of the extract. The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were checked to evaluate the antibacterial effect. The CFUs and survival rate of *E. faecalis* showed growth inhibition as the concentration increased. The MIC and MBC were found to be 2.5 and 30 mg/mL, respectively. *Chamaecyparis obtusa*extract with an antimicrobial effect on *E. faecalis*may be used as an irrigation agent in place of chemicals that cause resistance in endodontic treatment.

Keywords: Antimicrobial activity, *Chamaecyparis obtuse*, Enterococcus faecalis, Oral health.

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Introduction

There are over 500 species of bacteria in the human mouth, and they interact with one another as commensal bacteria and pathogens to maintain oral health balance [1]. As pathogenic bacteria can cause oral diseases and dental loss, much time and cost are required for their removal [2]. Pulpitis is an infectious disease caused by pathogenic bacteria most frequently generated in the oral cavity, along with dental caries and periodontal disease [3]. The normal pulp is mostly sterile because it is surrounded by dental hard tissues, making it difficult for bacteria to invade it. If trauma or oral disease develops in the oral cavity, however, the pulp may be exposed to bacteria, causing inflammation and progression to pulp necrosis [4]. Even if the pulp tissue is necrotic, bacteria can exist therein, so it is important to remove the bacteria that can cause pulpitis [5]. When bacteria grow in the root canal, they develop into apical lesions, so research on bacteria related to pulpitis is essential.

Early pulpitis mainly has a high proportion of facultative *streptococci*, but when pulpitis develops, 70-95% of the bacteria is anaerobic bacteria [4-9] and are commonly called "black-pigmented anaerobic rods." Among them, bacteria belonging to the genera *Porphyromonas* and *Prevotella* mainly appear [7,10], and they must be removed through endodontic treatment, but if they are not completely removed, *Enterococcus faecalis* (*E. faecalis*) will grow in the root canal [11,12].

E. faecalis causes opportunistic or nosocomial infections in the urethra, blood, endocardium, abdomen, bile ducts, and burn area [13,14], mucosal infections in patients with reduced immunity [15], and superinfection[16] in periodontitis. It is effectively removed by washing with NaOCl and chlorohexidine[17], which have been reported to be very effective when used as an intracanal medication by mixing chlorohexidine gel with chlorohexidine and Ca(OH)2 [16,19]. Unlike other bacteria, however, *E. faecalis* can penetrate deeply within a short period of time because invasion of the dentinal tubules is not inhibited by serum, and adhesion to collagen is increased [20,21]. Therefore, if *E. faecalis* is in the dentin, using Ca(OH)2 will have no effect on it, so its continuous survival is possible [22-24]. For this reason, as *E. faecalis*survives in the dentinal tubules for a long time, the antimicrobial action of root canal sealers is emphasized. In this study, Ca(OH)2 and ZOE-based root canal sealers were more effective than resin-based AH26 with hexamethylenetetramine added [25], but they were applied only for a short time, and it was reported that their antimicrobial effect was reduced when

they were applied for 7 days or more [26]. Moreover, *E. faecalis*that is resistant to vancomycin, which is used for bacteria that are resistant to multiple antibiotics, is emerging [27], and once one is infected by it, treatment is difficult because when it forms a biofilm in the oral cavity, it becomes more resistant to antibiotics. Therefore, the effectiveness of antibiotics for preventing or treating pulpitis and for preventing reinfection after endodontic treatment is temporary, so it is difficult to use them for a long time. Accordingly, it is necessary to develop an antimicrobial agent that is safe and has no side effects or resistance even when used for a long time. As the research on the utilization of natural extracts has been activated, various methods of using natural antimicrobial activity are currently being sought [28].

Chamaecyparis obtusa, which is effective against harmful bacteria in the oral cavity, is an evergreen coniferous tree of Oriental arborvitae and retinispora, which grows in Japan and Taiwan and produces a large amount of phytoncide with a unique fragrance on its stem [29]. Phytoncide is a volatile oil obtained from plant flowers, leaves, stems, roots, and resins, and is known to have antimicrobial effects against bacteria and fungi [30,31]. In particular, it has an antifungal effect against *Candida albicans*, which causes oral candidiasis [32]. While research on the antimicrobial effects of *Chamaecyparis obtusa* extract against causative bacteria of oral diseases has been actively conducted, antimicrobial agents that can be used for the purpose of preventing or inhibiting progress are continuously being studied, but the studies on *E. faecalis*, which causes pulpitis, are not sufficient [33-36]. Therefore, this study intended to identify the antimicrobial effect of *Chamaecyparis obtusa*extract against *E. faecalis*, a typical causative bacterium that causes pulpitis, and to suggest the possibility of using *Chamaecyparis obtusa*extract instead of chemicals for its endodontic treatment.

Materials and Methods

Clinical application *Chamaecyparis obtusa* preparation

The dried *Chamaecyparis obtusa* that was used in this study was purchased from Foodsynergy Co., Ltd. (Seoul, South Korea). After adding 70% ethanol to crushed Chamaecyparis obtusa, an extract was obtained at 60°C for 12 h. The extract was filtered using filter paper (Whatman No. 2, Tokyo, Japan), and the *Chamaecyparis obtusa* extract was concentrated using a rotary vacuum evaporator (N-1300E.V.S. EYELA Co., Tokyo, Japan) at 40°C. The concentrated *Chamaecyparis obtusa* was lyophilized using a freeze dryer (Ilshin Lab Co., South Korea).

Chemicals

E. faecalis(ATCC 4083) was used and grown in brain heart infusion (BHI; Sigma-Aldrich, St. Louis, MO, USA). It was cultivated overnight in liquid media incubated at 37°C. The cell was diluted into approximately 1×10^6 colony-forming units (CFUs) per milliliter.

Antimicrobial activity of *Chamaecyparis obtusa* extract

100 μL of cultured E. faecalis (1×10^5 CFUs/ml) was inoculated into a 24-microwell plate containing BHI broth in which *Chamaecyparis obtusa*extract was added at each concentration (0, 1.25, 2.5, 5, 10, 20, and 30 mg/ml). The total volume of each mixture was 1 ml. The microplate was incubated anaerobically for 24 h at 37°C, and then the mixture in each well was uniformly smeared in an agar medium and then cultured at 37°C for 24 h.

Measurement of the MIC and MBC of *Chamaecyparis obtusa* extract

The activity of *E. faecalis*was incubated in a BHI liquid medium for 24 h at 0, 1.25, 2.5, 5, 10, 20, and 30 mg/ml *Chamaecyparis obtusa*extract concentrations. After incubation for 24 h, the changes in the bacterial growth were determined by measuring the optical density (OD) at 660nm.

Statistical Analysis

Significance analysis of the inhibitory effect was carried out using Ver. 21.0 (SPSS Inc., Chicago, IL, USA). The difference in each concentration was evaluated through one-way analysis of variance (ANOVA), followed by the Ducan test ($p<0.05$).

Results

Antibacterial effects of *Chamaecyparis obtusa* extract

The antibacterial effect of *Chamaecyparis obtuse* extract against *E. faecalis* was evident with increasing concentration (Figure 1). As shown in Table 1, the death rate of *E. faecalis* was 1.9 ± 0.1 1010 at 0 mg/ml, 4.2 ± 0.3 108 at 1.25 mg/ml, 2.7 ± 0.4 107 at 2.5 mg/ml, 5.0 ± 0.2 105 at 5 mg/ml, 6.0 ± 0.3 103 at 10 mg/ml, 3.1 ± 0.0 101 at 20 mg/ml, and 0 at 30 mg/ml. In the change according to the concentration, there was a statistically significant evidence ($p<0.05$).

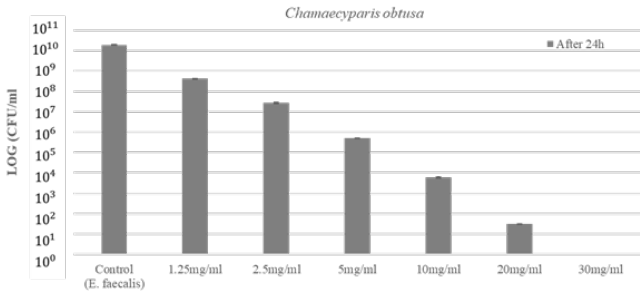

Figure 1. *Survival rate of Chamaecyparis obtusa extract against E. faecalis after 24 h.*

Table 1: *Changes in the growth of E. faecalis and p-value of the antibacterial activity of Chamaecyparis obtusa extract.*

Concentrations (mg/ml)			MIC			MBC		
			2.5 mg/ml			30 mg/ml		
Group	E. faecalis	1.25 mg/ml	2.5 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	ANOVA
After 24 h	(0 mg/ml)	4.2 ± 0.3 108b	2.7 ± 0.4 107c	5.0 ± 0.2 105c	6.0 ± 0.3 103c	3.1 ± 0.0 101c	0.0c	p-Value

Significant difference among the groups in one-way ANOVA. Different letters (a, b, and c) by the presented statistically significant result of the posthocDucan (: $p<0.05$).

Growth inhibitory effect of *Chamaecyparis obtusa* extract

The absorbance was measured for each concentration to observe the antimicrobial effect of *Chamaecyparis obtusa*extract (Table 1). At 24 h, the MIC and MBC were determined to be 2.5 and 30 mg/ml, respectively.

Discussion

Pulpitis is caused by bacteria penetrating the root canal. There are mechanical methods of removing it, such as root canal formation and chemical methods using root canal cleaning fluid [37,38]. After such endodontic treatment is completed, 40-50% of patients develop postoperative lesions radiographically, indicating that perfect endodontic treatment is difficult in reality [12, 39-41]. The reasons for this include infection in the root canal, infection outside the root canal; the formation of cholesterol crystals in the cyst, or foreign-body reactions, but the main cause of failure is the bacteria that survive in the root canal. Endodontic treatment usually requires a month-long treatment, during which time *E. faecalis*proliferates due to the temporary sealing [42]. It has been found to be present at high concentrations in the root canal of the teeth, with persistent periapical lesions, and is believed to be an important bacterium causing endodontic treatment failure [11,43]. In addition, its ability to form biofilms in the root canal has been reported as a cause of endodontic treatment failure due to resistance to drugs [44-46].

E. faecalis is an intestinal bacterium that causes opportunistic infections in various parts of the body [13, 47], but there are very few in the oral cavity as it temporarily stays therein in the process of migration to the gastrointestinal tract. If it invades the root canal, however, due to insufficient sterile treatment during the endodontic treatment process [43], it is resistant to the root canal cleaning fluid [22,23], root canal sealer [48], antibiotics [17], etc., consequently becoming the most important bacterium causing endodontic treatment failure [11,12]. *E. faecalis* is not only resistant to various antibiotics, such as penicillin, ampicillin, clindamycin, metronidazole, and tetracycline[24]; strains resistant to vancomycin have emerged of late [49], and super infection has appeared [50], causing clinically very serious problems. In a study by Chung et al. [51], it was observed that some of the bacterial isolates of periodontal disease, for which the presence or absence of resistant genes was examined, delivered resistant genes to *E. faecalis* strains. These results suggest that if *E. faecalis* is present during root canal infection, the prevention and treatment of such infection may become more difficult if resistance to multiple antibiotics is obtained by actively exchanging new resistance with the existing antibiotic resistance. Therefore, there is an urgent need to develop natural drugs that are efficient and safe to use to replace antibiotics. Therefore, *E. faecalis*,the most frequently bacterium when endodontic treatment fails, is highly resistant to several types of antimicrobics and antibiotics [17,22,23,50], and unlike other bacteria, it can survive sufficiently even if it exists alone. Due to its excellent ecological adaptability, it is difficult to remove fundamentally with the conventional treatments and therapeutic drugs [52].

This study thus investigated the usability of *Chamaecyparis obtusa*extract by observing its antimicrobial effect against *E. faecalis*. As a result, *Chamaecyparis obtusa*extract showed an excellent antimicrobial effect against *E. faecalis*with increasing concentration. It was found in this study that the growth of bacteria was effectively suppressed at 2.5 mg/ml. It was confirmed that the bacteria were completely killed at 30 mg/ml, the MBC where no colonies were confirmed and where a distinct antimicrobial effect was shown. These results were also confirmed by the CFU results.

The extracts from Vernonialasiopus, Bulbinefrutescens, and Tagetesminuta showed antimicrobial activity against *E. faecalis*[53]. According to the study by Duailibe et al. [54], the S. paniculatum Linn extract exhibited inhibitory effects on *E. faecalis*up to a 7.81 mg/mL concentration. The present study confirmed, however, that *Chamaecyparis obtusa*has an MIC of 2.5 mg/ml, thereby having an inhibitory effect at a lower concentration compared to S. paniculatum Linn extract. There has been no prior research on *E. faecalis*using *Chamaecyparis obtusa* extract, so there are limitations in comparison, but considering the reality that many pathogens are resistant to the existing chemical antibiotics, the research investigating the applicability of natural substances in the treatment of pulpitis is important. Therefore, it is considered that *Chamaecyparis obtusa*extract can be developed as a treatment for pulpitis due to its high antimicrobial activity against intracanal infection.

Conclusion

*Chamaecyparis obtusa*extract can be utilized as a bio-friendly natural antibiotic that can be safely used as a

perfect treatment for pulp disease.

Conflict of interest

The authors report no conflicts of interest related to this study. The author does not have any financial interest in the companies whose materials are included in the article.

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