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ORIGINAL ARTICLE

Antibacterial Effects of Cuminum cyminum Extract Against *Enterococcus Faecalis* Biofilms From Clinical Isolates

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Abstract

Objective: To compare the antibacterial efficacy of Cuminum cyminum (cumin) extract and 2% chlorhexidine. **Material and Methods:** *E. faecalis* was isolated from non-vital teeth with chronic apical abscess. Samples were then bred in the ChromAgar medium. Subsequently, *E. faecalis* bacteria's DNA extraction was performed. DNA was then amplified by conventional PCR, and the product was run on an electrophoresis gel. Subsequently, we extracted *Cuminum cyminum* seeds using the steam distillation technique. The extract was diluted at various concentrations: 0.2, 0.5, 0.7, 1.0, and 1.2 mg/mL. The extract's antibacterial effect was evaluated using an ELISA reader with optical density. Specifically, we assessed the turbidity of *E. faecalis* in biofilms following immersion in antibacterial agents. **Results:** In the clinically isolated *E. faecalis* group, the OD values of 0.7 and 1.0 mg/mL cumin extracts were significantly different from that of 0.2 mg/mL cumin extract. A significant difference was also observed between the OD values of 1.0 mg/mL cumin extract and 2% CHX ($p<0.05$). **Conclusion:** The antibacterial effect of 1.0 mg/mL *Cuminum cyminum* extract had higher efficiency than 2% chlorhexidine against *E. faecalis* biofilms from clinical isolates.

Keywords: Plants, Medicinal; Biofilms; Chlorhexidine; Enterococcus faecalis.

Introduction

Bacteria within biofilms have an inherently increased resistance to antimicrobial agents compared to the same bacteria grown under planktonic conditions [1]. Mature biofilms have 10–1000 times higher tolerance to antibiotics than planktonic bacteria [2]. Numerous studies have shown that *Enterococcus faecalis* is the most common species in persistent endodontic infections, displaying a prevalence of 24%-77% [3]. Furthermore, the virulence of *E. faecalis* contributes to its survival in minimal environmental conditions [4]. Therefore, effective and safe antibacterial agents are necessary to eliminate resistant bacteria in root canal systems.

In endodontics, the most common disinfection materials for root canal cleansing are synthetic. Specifically, the most widely used irrigation material is 2% chlorhexidine (CHX), which is considered the gold standard in eliminating *E. faecalis* from the root canal system. Importantly, 2% CHX has been shown to eliminate *E. faecalis* present in biofilms [1,5]. However, the effectiveness of CHX depends on its concentration. Importantly, while increasingly high concentrations of CHX increase its effectiveness, they also increase its toxicity [6,7]. A previous study that examined the effects of various CHX concentrations (0.06%, 0.12%, 0.2%, 1%, and 2%) on odontoblast-like cells (MDPC-23 cells) showed that all concentrations had cytotoxic effects and were linked to decreased cell metabolism ranging between 61% and 70% as the dose increased [8].

Because of an increase in bacterial resistance to synthetic disinfection agents (CHX being one of them) and the side effects associated with such agents, novel research efforts have been focusing on identifying alternative disinfection agents [9-11]. Numerous studies have suggested that *Cuminum cyminum* (cumin) possesses various therapeutic properties, including antioxidant, antibacterial, antifungal, analgesic, and anti-inflammatory properties. *Cuminaldehyde*, the primary chemical constituent of *Cuminum cyminum*, plays a role in its antibacterial effect [12-14]. Of note, several studies have proven the effective antibacterial action of cumin extract in the treatment of urinary tract infections caused by *E. faecalis* [13,15]. The antibacterial action of cumin extract on *E. faecalis* (AGH 011) was tested by measuring the zone of inhibition and kill. After 24 h of incubation, the zones of inhibition of cumin extract and CHX gel against *E. faecalis* were found to be 28.75 mm (13.74 mm) and 18.75 mm (2.49 mm), respectively. The authors found that the antibacterial effect of cumin extract at a concentration of 0.7 mg/mL was more effective than that of 2% CHX gel. It was also associated with lower toxicity [16].

To date, there are no studies that have tested the antibacterial effects of cumin extract against *E. faecalis* biofilms from endodontic clinical isolates. Current research efforts have been focusing on investigating the antibacterial effects of cumin extract on *E. faecalis* isolated from the digestive system [13,14,17]. Some authors examined the differences in *E. faecalis* isolated from endodontic infections versus urinary tract infections and observed that *E. faecalis* isolated from endodontic infections were characterized by a higher number of virulence factors than *E. faecalis* isolated from the digestive tract, making it less susceptible to antibacterial agents[18]. Therefore, in

the present study, we aimed to analyze the antibacterial effects of cumin extract versus 2% CHX on *E. faecalis* isolated from teeth with periapical lesions.

Material and Methods

Study Design

The study conducted was a laboratory experimental study, which consisted of the preparation of cumin extract, *E. faecalis* isolation, antibacterial efficacy testing with crystal violet assay, and analysis.

Preparation of the Cumin Extract

The cumin extract was obtained by the steam distillation technique. Subsequently, the chemical constituents of the extract were analyzed using gas chromatography-mass spectrometry (GCMS). The cumin extract was placed in a black bottle and stored in a refrigerator. The cumin extract was subsequently diluted with dimethyl sulfoxide (DMSO) to the following concentrations: 0.2, 0.5, 0.7, 1.0, and 1.2 mg/mL.

Isolation of *E. faecalis*

The sample of the study was obtained from the Conservative Dentistry Clinic of Faculty of Dentistry Universitas Indonesia patients who came for endodontic treatment. Following the diagnosis, patients received a detailed explanation about the research and signed informed consent. A subset of the samples was isolated from non-vital teeth with chronic apical abscess. Samples were then bred in the ChromAgar medium. Subsequently, *E. faecalis* bacteria's DNA extraction was performed. DNA was then amplified by conventional PCR, and the product was run on an electrophoresis gel. Following is the primer set used to identify *E. faecalis* DNA: *5'* TACTGACAAACCATTCATGATG *3'* forward; *5'* AACTTCGTCACCAACGCGAAC *3'* reverse.

Antibacterial Efficacy Testing Using Crystal Violet Assay

E. faecalis biofilms were grown in 96-well plates. Two hundred microliters of *E. faecalis* solution was applied to each well-plate, and they were incubated at 37°C for 24 h. Subsequently, the well-plates were washed with PBS and then covered with test materials either containing cumin extract or 2% CHX (GLUCO-CheX). The biofilms were then incubated with the test materials for 15 min at 37°C. We obtained the optical density (OD) value by using an ELISA reader with a wavelength of 450 nm for 10 s. We then calculated the volume of clinical isolates tested into a sterile tube of 1×10^6 cells/mL. PBS was used to wash each well-plate, and 200 µL of 0.1% crystal violet solution was then poured onto the well-plates and incubated for 15 min. The crystal violet solution was then removed, and the plates were washed with PBS. Following this, we added 200 µL of 95% ethanol to each well-plate. The OD value was tested using an ELISA reader machine set at 450 nm and a shaker of the duration of 10 s.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics (IBM Corp., Armonk, NY, USA). Statistical analysis was performed using one-way ANOVA, with a significance level set at 5%.

Ethical Aspects

This study was approved by the Ethics Committee of Universitas Indonesia, Faculty of Dentistry (Ethics No. 106/Ethical Approval/FKGUI/XI/2017 – Protocol No. 051181017).

Results

From cultures of *E. faecalis bacteria* on selective *chromAgar*, bacterial growth was found in 4 out of 7 *chromAgars*. The bacterial growth was observed as green colonies on the agar, and bacterial DNA was extracted using PCR and electrophoresis. Electrophoresis showed the presence of bright white bands located parallel to the *E. faecalis* markers.

Cumin extracts were obtained by means of a distillation technique (steam distillation) which produced 100% of cumin extract. Before dilution, the chemical constituents of the cumin extract were analyzed using GCMS. Cuminaldehyde was the predominant compound, accounting for 61.65% of the extract (Table 1). The cumin extract was then diluted with DMSO to obtain the following concentrations: 0.2, 0.5, 0.7, 1.0, and 1.2 mg/mL.

Table 1. Composition of Cumin extracts' chemical compounds.

Compounds	Percentage
Cuminaldehyde	61.65
Cumene	10.79
p-Cymene	6.61
β-Pinene	2.64
Acetic acid	1.93
p-Cymen-7-ol	1.74
Pentanoic Acid	1.66
Formic acid	1.23
γ-Terpinene	1.12
Others	10.63

In the present study, *E. faecalis* biofilms were exposed to various concentrations of cumin extract (0.2, 0.5, 0.7, 1.0, and 1.2 mg/mL) and 2% CHX. We used crystal violet assay with an ELISA reader as an antibacterial test method. After exposing *E. faecalis* biofilms to the test materials for 15 min at 37°C, crystal violet coloration was applied, and the solution's turbidity was observed with an ELISA reader. The solution's turbidity values were marked by the OD values. The higher the OD value, the more turbid the solution was, meaning an increased number of bacteria in the solution. In such cases, we deduced that the test material had poor antibacterial ability. The average results of the OD values are summarized in Table 2.

Table 2. *E. faecalis* bacterial biofilms' mean values of the optical density values following exposure to Cumin extract and 2% CHX.

Group	N	Mean ± SD	95% CI	
			Lower Limit	Upper Limit
0.2 mg/ml Cumin extract	6	0.0630 ± 0.0021	0.0577	0.0683
0.5 mg/ml Cumin extract	6	0.0563 ± 0.0015	0.0526	0.0601
0.7 mg/ml Cumin extract	6	0.0552 ± 0.0013	0.0518	0.0584
1.0 mg/ml Cumin extract	6	0.0516 ± 0.0013	0.0484	0.0549
1.2 mg/ml Cumin extract	6	0.0563 ± 0.0008	0.0541	0.0586
2% CHX	6	0.0619 ± 0.0025	0.0553	0.0683

After obtaining the OD values, data were analyzed using a one-way ANOVA test with an SPSS 24.0. In the clinically isolated *E. faecalis* group, the OD values of 0.7 and 1.0 mg/mL cumin extracts were significantly different from that of 0.2 mg/mL cumin extract. A significant difference was also observed between the OD values of 1.0 mg/mL cumin extract and 2% CHX (Table 3).

Table 3. Optical density's dignificance dalues of clinically isolated *E. faecalis* bacterial biofilms after exposure to Cumin extracts and 2% CHX.

Groups	Cumin Extract					2% CHX
	0.2mg/ml	0.5mg/ml	0.7mg/ml	1.0mg/ml	1.2mg/ml	
0.2 mg/ml Cumin extract	-	0.129	0.037*	0.001*	0.129	1.000
0.5 mg/ml Cumin extract	0.129	-	1.000	0.874	1.000	0.41
0.7 mg/ml Cumin extract	0.037*	1.000	-	1.000	1.000	0.129
1.0 mg/ml Cumin extract	0.001*	0.874	1.000	-	0.874	0.003*
1.2 mg/ml Cumin extract	0.129	1.000	1.000	0.874	-	0.41
2% CHX	1.000	0.41	0.129	0.003*	0.41	-

*One-Way ANOVA significance test between groups with $p<0.05$.

Discussion

The present study represents a preliminary evaluation aimed at determining the possible antibacterial effects of cumin extract on *E. faecalis* biofilms from endodontic clinical isolates. Of all the obtained samples, *E. faecalis* were found in 57% of the sampled patients. While it has been shown that *E. faecalis* is frequently found in persistent endodontic infections[3,19,20], *it* also appears to be one of the most common bacteria in primary endodontic infections. These findings are in line with previous data that demonstrated that *E. faecalis* was present in 55% of primary endodontic infections with open cavities [21].

The cumin extract used in this study contained a large amount of *cuminaldehyde* (61.65%). Other constituents of the extract included *cumene; p-cymene, β-pinene, acetic acid, p-cymen-7-ol*, and *γ-terpinene*. These findings are in line with previous studies, although with different amounts [14,16]. The cumin extract contains numerous active chemical compounds that play a role in its antibacterial effect [16,22]. Furthermore, cumin has been shown to possess hydrophobic characteristics capable of degrading the lipids contained in the bacterial cell walls and the mitochondria, in turn damaging the structure of the bacterial cells [16]. Numerous studies have examined the antibacterial effect of cumin extract on several human infectious bacteria, with a focus on those resistant to antibiotics [12-14].

To date, the optimal dosage reference for cumin extract as an antibacterial agent is not known. To this end, in the present study, we examined the effects of various concentrations of cumin extract on *E. faecalis* biofilms. In a previous study on *E. faecalis* in planktonic forms, it was shown that cumin extract at a concentration of 0.7 mg/mL had better antibacterial effects than 2% CHX gel, with lower toxicity [16]. Therefore, here we added several other concentrations (0.2, 0.5, 1.0, and 1.2 mg/ml) to identify an effective dose on *E. faecalis* biofilms.

In the present study, 2% CHX was used as a positive control. Currently, CHX has been widely used both as an endodontic irrigation solution and as an intracanal medicament [6,23]. The previous study suggested that 2% CHX and 5.25% NaOCl have comparable antibacterial efficacy [1]. According to the literature, 2% CHX can eliminate *E. faecalis* in 1 min [6].

E. faecalis biofilms used in this study were 24 hours old. In 2004, some authors examined the development of *E. faecalis* biofilms by using scanning electron microscopy (SEM) and observed that bacterial cell adhesion occurred in the timespan of 2 h and the continuous formation of bacterial microcolonies within 8 h. Subsequently, in the timespan of 20 h, *E. faecalis* biofilms were formed [24].

Previous research aimed to find an alternative substance for root canal medicament using a 24-hour incubation [16]. On the contrary, in the present study, we performed a 15-min incubation, aimed at analyzing the potential of cumin extract as an alternative to current root canal irrigation materials. We chose this incubation timing following a previous study that analyzed the antibacterial effect of 2% CHX solution on biofilms of various bacteria, including *E. faecalis*. The authors found that 2% CHX could achieve complete elimination of bacteria after 15 min [1]. It was shown that more than 5 min was required to obtain the maximum binding capacity of antiseptic particles to bacterial cell walls [25]. Similarly, other researchers observed that antibacterial agents had to be in contact with bacteria in biofilms for a minimum of 10 min to successfully eliminate them [26].

In Table 2, we show that the OD mean values for some concentrations of cumin extract are lower than those for 2% CHX. The cumin extract at a concentration of 1.0 mg/mL had the lowest mean value of OD, specifically of 0.0516 (±0.0013). In addition, it had the lowest turbidity, the least number of bacteria, and the best antibacterial effect. This value was lower than the OD mean value of the 2% CHX group, which was 0.0619 (± 0.0025). On the contrary, the cumin extract group at a concentration of 0.2 mg/mL had the highest OD mean value, specifically 0.0630 (± 0.0021). Furthermore, our results show that the white cumin extract group at a concentration of 1.0 mg/mL had a significantly different OD value from that of the 2% CHX group. Based on these results, it can be concluded that the antibacterial effect of the cumin extract at a concentration of 1.0 mg/mL was better than that of the 2% CHX against *E. faecalis* from clinical isolates.

Cuminaldehyde is an active compound of cumin extract, which is present in the highest concentration and with potential antibacterial effects [12-14]. The concentration of *cuminaldehyde* found in the present study was 61.65%. This finding was in line with studies that suggested that cumin extract had the highest percentage content of 35%-63% [14]. *Cuminaldehyde*, an aromatic

volatile component present in cumin extract, is an oxidized aldehyde monoterpene compound ($C_{10}H_{12}O$) [12,27]. This compound changes the outer layer of bacterial cells, thereby inhibiting ion transport in and out of the cell. Ultimately, this process interferes with the activity of bacterial enzymes [28]. Dialdehyde and glutaraldehyde have the advantage of working well in acidic and alkaline conditions. Once inside the cell, aldehyde could interact with bacterial DNA and eventually interfere with bacterial growth. While the actual process of aldehyde's penetration into bacterial cells has not been fully clarified, it has been suggested that it passively diffuses through the plasma membrane [28].

Among the various doses analyzed in this study (i.e., 0.2, 0.5, 0.7 1.0, and 1.2 mg/ml), the most effective dose against *E. faecalis* biofilms was 1.0 mg/mL. The wide range of active components in herbal extracts capable of influencing each other determined these results, either synergistically or antagonistically [16]. We hypothesized that besides the increasing doses, the active components with antagonistic effects would have stronger outcomes that might cause a decrease in their antibacterial function. Therefore, it was critical to acknowledge the fact that cuminaldehyde is the active component with the largest percentage of cumin extract. Our findings suggest that *cuminaldehyde* fractionation could be further analyzed in future studies. *Cuminaldehyde* fractionation isolated from cumin extract should confirm the antibacterial efficacy of this active component.

Conclusion

The cumin extracts demonstrated antibacterial effects against *E. faecalis* biofilms isolated from clinical patients. Cumin extract at a concentration of 1.0 mg/mL has better antibacterial efficacy than 2% CHX against *E. faecalis* biofilms isolated from patients.

Authors' Contributions: RA perfomed the experiments, analysis and wrote the manuscript, AM designed the study, interpretation and criticaly revised the manuscript, SUD contributed to conception and data design, MU contributed to analysis and critically revised the manuscript.

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Conflict of Interest: The authors declare no conflicts of interest.

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