

## GARLIC EXTRACT EFFICACY AGAINST THE VIABILITY OF *ENTEROCOCCUS FAECALIS* (IN VITRO)

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### ABSTRACT

**Objective:** *Enterococcus faecalis*, Gram-positive facultative anaerobe capable of invading the dentinal tubules, is resistant to several irrigating solutions and intracanal medications. Motivated by the antibacterial effect exhibited by garlic extract against a wide variety of Gram-positive and Gram-negative bacteria, as well as anaerobic bacteria such as *Lactobacillus* and *E. faecalis*, we aimed in this study to analyze the efficacy of garlic extract against the viability of *E. faecalis* from clinical isolates of nonvital primary root canals.

**Methods:** We used MTT assay to determine the viability of *E. faecalis* after exposure to increasing concentrations of garlic extract (10%, 25%, 50%, and 100%) and chlorhexidine (CHX) 2% as a positive control.

**Results:** We performed the analysis using the Kruskal–Wallis test and the *post hoc* test for multiple comparisons at a 0.05 significance level. At all concentrations, garlic extracts decreased the viability of *E. faecalis*. We found no significant differences between the viability values for 25%, 50%, and 100% garlic extracts ( $p > 0.05$ ); however, they were significantly different from the 10% extract and CHX 2% ( $p < 0.05$ ), both with a lower viability values.

**Conclusion:** Our results showed that garlic extract was effective in decreasing the viability of *E. faecalis*.

**Keywords:** Garlic extract, viability, *Enterococcus faecalis*.

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### INTRODUCTION

*Allium sativum*, or garlic, is a plant proven to have drug efficacy and to possess antibacterial, antifungal, and antiviral properties. Garlic extract reportedly has antibacterial effects against a wide range of bacteria, including *Escherichia coli*, *Salmonella enteric*, *Shigella*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus* (*S. faecalis*, *S. mutans*, and *S. pyogenes*), *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*. The crushing or chopping of cloves of garlic releases their antibacterial activity, which derives from the allicin compound through the enzymatic activity of alliinase (cysteine sulfoxide lyase) in alliin. Allicin and other thiosulfinates are believed to be associated with the therapeutic effects of garlic [1-3].

Root canal infections of primary teeth, whether symptomatic or asymptomatic, are caused by microbes found in the root canal. The microorganisms found in the root canals of primary teeth, like those of the permanent dental root, are aerobic and nonaerobic bacteria and facultative bacteria [4,5]. da Silva *et al.* found aerobic and anaerobic microorganisms, black-pigmented bacilli, *Streptococci*, and Gram-negative aerobic rods in the root canals of nonvital primary teeth [6]. Other studies found *Enterococcus* spp. and *Porphyromonas gingivalis* to be the predominant bacteria found in the root canals of nonvital primary teeth [4,7]. *E. faecalis* were anaerobic facultative Gram-positive bacteria with powerful virulence factors including the ability to compete with other microorganisms, invade the dentinal tubules, and survive nutritional deficiencies. Adhering to the root canal walls, accumulating to form biofilms, and so becoming more resistant to irrigation substances and intracanal medicaments, they were consequently often associated with persistent infections and root canal treatment failures [8-10].

Root canal treatments conducted in primary teeth are intended to preserve them until exfoliation and replacement by permanent teeth. Root canal clearance of primary teeth aims to remove the necrotic tissue as far as possible in the root canal without disturbing the emergence of the permanent tooth buds [11], a process known as a chemomechanical preparation, an active chemical solution or irrigation solution used in conjunction with mechanical instrumentation in the root canal. Most microorganisms present in root canal infections of primary teeth were found in the apical ramifications and dentinal tubules [5]. Because the infected primary teeth, as with the permanent teeth, could store microorganisms in the dentinal tubule, the use of chemicals was required to help disinfect the area. Throughout the instrumentation, irrigation solutions could remove necrotic and contaminated materials, disinfect the root canals and dentinal tubules, and eliminate bacteria [12]. Commonly used root canal irrigants include sodium hypochlorite (NaOCl), chlorhexidine (CHX), and other chemicals that could have a toxic effect on the periapical tissues and permanent tooth buds when pushed to the apex [5].

The increased resistance to antibiotics of microbes and toxicity of antimicrobial agents has engendered a need for alternative, nontoxic, effective, and readily available agents. Many natural plant extracts could be applied as effective root canal irrigation solutions. The main advantages of herbal irrigants are their safety, ready availability, durability, cost-effectiveness, and, to date, lack of evidence of bacterial resistance [12]. Several *in vitro* studies had shown that herbal plants, one of which is the extract of garlic (*A. sativum*), possess promising properties as antibacterial agents, as evidenced by several *in vitro* studies. We aimed in our study to analyze the efficacy of garlic extract at various concentrations against the viability of *E. faecalis* obtained from the clinical isolates of nonvital primary root canals.

## METHODS

Our laboratory experimental study comprised sample collection, *E. faecalis* isolation, preparation of garlic extract, viability tests with MTT assay, and statistical analysis.

### Sample collection

We enrolled patients aged 4–8 years who attended the pediatric dental clinic of Universitas Indonesia and were diagnosed with nonvital primary tooth due to caries with or without periapical infection. The Ethics Committee of the Faculty of Dentistry, Universitas Indonesia, approved all clinical procedures. Before the commencement of the study, we obtained informed consent from each parent/guardian, together with a detailed medical and dental history. We excluded patients who had received antibiotic therapy in the past 3 months, or were suffering from a systemic disease, from this study. We included patients who fulfilled the criteria of patient and parent cooperation, teeth with intact roots or <1/3 of physiological root resorption, no root canal intervention prior to the study, no root canal obliteration, and the remaining crown allowing isolation with a rubber dam and further restoration.

To ensure antisepsis of the oral cavity, patients rinsed for 1 min with 10 mL of 0.12% CHX; we then placed a rubber dam. After the removal of carious tissue, we accessed the root canal with high-speed sterile diamond burs. We collected samples with a sterile #15 K-file to agitate canal contents for 60 s. We introduced the file to a level approximately 1 mm short of the tooth apex based on the diagnostic radiographs, or to the limit of the physiological root resorption, and used a gentle filing motion. We then placed two sequential paper points to the same level and used them to soak up the fluid in the canal, holding each paper point in position for 60 s. If the root canal was dry, we introduced a small amount of sterile saline solution into the canal to ensure viable sample acquisition. We never used chemically active irrigants before sampling. We then transferred the paper points and K-file to sterile Eppendorf tubes containing 1 mL of brain–heart infusion (BHI) broth. We stored the samples at 4°C before culture.

### *Enterococcus faecalis* isolation

After shaking the tubes containing samples in a vortex mixer, we transferred 50 µL and plated it on the Chromagar plates, which we incubated under anaerobic condition at 37°C for 24 h. Having confirmed the culture visually, we transferred it to 7 mL BHI broth by taking one colony of bacteria from the Chromagar plate. We then incubated the plates under anaerobic conditions at 37°C for 24 h. We extracted the bacterial DNA from *E. faecalis* and performed DNA amplification by conventional polymerase chain reaction (PCR). The primer pair used to detect *E. faecalis* was 5'TACTGACAAACCATTCATGATG 3' forward and 5'AACTTCGTCACCAACGCGAAC 3' reverse. We performed amplification with a thermal cycle as follows: denaturation at 94°C for 15 min, 40 cycles of denaturation at 94°C for 20 s, annealing at 68°C for 45 s, extension at 72°C for 15 s, final phase at 72°C for 7 min, and cooling phase at 4°C. We conducted DNA identification by the electrophoresis process.

### Preparation of garlic extract

We obtained garlic (*A. sativum* L.) from a traditional market in Jakarta and used an extraction method based on Fujisawa *et al.* We crushed 40 g of garlic cloves with a utility garlic crusher and collected the juice and the debris of the garlic in a centrifuge tube, adding 40 mL of solvent

(ethanol 20%). After shaking the tube for 30 min, we allowed it to stand for 10 min at room temperature and then centrifuged it at 9000 rpm for 10 min, after which we filtered the garlic juice with filter paper to separate the supernatant. To achieve concentrations of 10%, 25%, and 50%, we diluted the extract with sterilized distilled water.

### Viability test using MTT assay

We homogenized the *E. faecalis* samples in BHI broth in a vortex mixer and added 100 µL of bacteria suspension to each well in a 96-well microplate, which we then covered with a lid and incubated under anaerobic condition at 37°C for 24 h. Then, we rinsed each well with sterile phosphate-buffered solution (PBS). We proceeded to expose the *E. faecalis* biofilm to 100 µL of garlic extract at different concentrations and 2% CHX as a positive control. We also prepared a negative control (medium only). We sealed the microplate and incubated it under anaerobic condition at 37°C for 24 h. After incubation with garlic extract, we rinsed each well with sterile PBS and added 50 µL of MTT solution (5 mg/mL) to the experimental wells, covered the plate with aluminum foil to attain a dark environment, and incubated it under anaerobic conditions at 37°C for 3 h. Then, we added 100 µL of acidified isopropanol to each experimental well and placed them on an orbital shaker at 50 rpm for 1 h. We read the absorbance value using an enzyme-linked immunosorbent assay reader with a wavelength of 490 nm. We used the optical density of the treatment and control groups in the following formula to calculate the cell viability percentage score:

$$\frac{\text{OD}_{\text{treatment}}}{\text{OD}_{\text{negative control}}} \times 100\%$$

### Statistical analysis

We tested all results by the Kruskal–Wallis statistical test with a significance level of  $p < 0.05$ .

## RESULTS

We obtained clinical samples from nonvital primary root canals. We detected *E. faecalis* from the presence of greenish-blue colonies in selective chromogenic medium and white bands obtained through the electrophoresis test using the conventional PCR method. We performed the statistical analysis using the Kruskal–Wallis test to analyze the difference in the viability value of *E. faecalis* against garlic extract at various concentrations.

Table 1 shows a decrease in the viability value of *E. faecalis* with the increase in garlic extract concentration. The Kruskal–Wallis test indicated that the viability values of at least two groups differed significantly. We conducted a *post hoc* Mann–Whitney test to identify the differences between treatment groups.

Table 2 shows the *post hoc* analysis results demonstrating statistically significant differences with regard to the viability values of *E. faecalis* between the negative control group versus the positive control and the entire garlic extract concentration group; the positive control versus groups of 25%, 50%, and 100% garlic extract; and the group of 10% garlic extract versus groups of 25%, 50%, and 100% garlic extract. However, we found no significant differences between the groups of 25%, 50%, and 100% garlic extract

**Table 1: Viability values of *Enterococcus faecalis* after administration of garlic extract at various concentrations**

Treatment group	n	Viability (%) Median (minimum—maximum)	p
Negative control	5	60.88 (54.12–191.24)	<0.001*
Positive control (2% CHX)	5	28.52 (24.18–30.17)	
10% garlic extract	5	16.73 (13.35–44.70)	
25% garlic extract	5	5.17 (4.94–7.45)	
50% garlic extract	5	4.71 (3.89–7.27)	
100% garlic extract	5	4.62 (4.21–6.17)	

Kruskal–Wallis analysis: \*Significance level based on  $p < 0.05$

Table 2: Post hoc analysis of viability values of *Enterococcus faecalis* in various concentrations of garlic extract

Test material	Garlic extract				2% CHX	Negative control
	10%	25%	50%	100%		
10% garlic extract		0.009*	0.009*	0.009*	0.295	0.009*
25% garlic extract	0.009		0.347	0.347	0.009*	0.009*
50% garlic extract	0.009*	0.347		0.917	0.009*	0.009*
100% garlic extract	0.009*	0.347	0.917		0.009*	0.009*
2% CHX	0.295	0.009*	0.009*	0.009*		0.009*
Negative control	0.009*	0.009*	0.009*	0.009*	0.009*	

Post hoc Mann-Whitney analysis. \*Significance level based on  $p < 0.05$ . CHX: Chlorhexidine

## DISCUSSION

We conducted this study to analyze the effectiveness of garlic extract against the viability of *E. faecalis* from clinical isolates. Our detection of *E. faecalis* in the primary teeth corresponded to a study that found *E. faecalis* to be the most common bacterium found in the root canals of nonvital deciduous teeth [7]. *Enterococcus* is a Gram-positive bacterium that may appear individually, in pairs, or as a type of facultative anaerobic bacteria (having the ability to grow in existing conditions and without oxygen) in a short-chain form. These bacteria were considered to be normal microbes commonly found in individuals and located principally in the digestive tract, female organs, and, at a lower frequency, the oral cavity. Only in recent years, *E. faecalis* has become a focus in health and dentistry. *E. faecalis* virulence factors include a lysis enzyme, cytolysin, aggregate substance, pheromone, and lipoteichoic acid. These factors influence the adhesion to the host cell, the release of proteins to compete with other bacteria, and altered host response. The persistence of *E. faecalis* located in the root canals is due to the presence of proteinases, gelatinases, and collagen proteins that enhance dentine bonds. These bacteria can survive for long periods in conditions of starvation until adequate nutrition becomes available. *E. faecalis* adheres to the root canal walls, accumulates, and forms biofilms, such that the bacteria are 1000 times more resistant than planktonic forms to phagocytosis, antibodies, and antimicrobials [8-10]. The ability of *E. faecalis* to form biofilms causes resistance to some commonly used irrigation solutions and intracanal medicaments [10].

As the antibacterial test in this study, we used the MTT assay. We used the popular method to estimate the metabolic activity of living cells. This test is based on the enzymatic reduction of a light-colored tetrazolium salt into a purple-bluish, spectrophotometrically quantifiable formazan. The absorbance value obtained is proportional to the number of living cells. The MTT assay could serve as one of the antibacterial test methods and could be an alternative or additional method for determining minimum inhibitory concentration, that is, the lowest concentration able to withstand bacterial growth. This method avoided interference with the results by dead bacterial cells. The MTT assay could also serve as an alternative method of microbial biofilm quantification. Unlike crystal violet staining, MTT made it possible to localize and quantify bacteria, particularly living cells [13].

Our results implied that various concentrations of garlic extract effectively reduced the number of viable *E. faecalis* bacteria *in vitro*. The highest viability value was found in the 10% garlic extract. This accorded with a previous study confirming the weak antibacterial effect of 10% garlic extract against *E. faecalis* [14]. According to Lee *et al.*, the minimum inhibition rate of garlic extract against the growth of *E. faecalis* was 12.8%; this accounted for the limited antibacterial efficacy of the 10% garlic extract in reducing the viability of *E. faecalis* [15]. Our results, in accordance with previous studies, demonstrated that the viability value would decrease with increasing concentrations [14,15]. The antibacterial activity of garlic resided in the allicin compound through the enzymatic activity of alliinase (cysteine sulfoxide lyase) in alliin after crushing or chopping cloves of garlic [1-3]. Allicin inhibited RNA synthesis and partially inhibited DNA and protein synthesis. The inhibition or limiting of RNA synthesis would greatly compromise

protein synthesis, which would be halted during each stage due to the absence of mRNA, rRNA, and tRNA. Without the production of amino acids and proteins, the growth and development of the organism would not occur. Allicin would also affect lipid synthesis, thereby disturbing other parts of the cells. Principal among these effects would be that the phospholipid layers of Gram-positive and Gram-negative bacterial cell walls could not be properly formed [16].

We extracted the garlic using an unheated maceration method with 20% ethanol solvent. This was in accordance with previous literature, suggesting that the best way to extract garlic was to avoid the use of high temperatures, which were damaging to the allicin compound and deactivated other important compounds. Chavan stated that the therapeutic nature of garlic was destroyed through warming. Allicin was an unstable component formed from the enzymatic action of alliinase and discharged at temperatures above 65°C [15]. Allicin could be more efficiently extracted with ethanol solution than with water, but the levels decreased gradually at room temperature, and the majority of allicin disappeared within a few days, especially in 100% ethanol and water. To maintain the allicin level for approximately 2 weeks at room temperature, the use of 20% ethanol as a solvent seemed to be more appropriate. In the process of extracting allicin from garlic, the use of ethanol solutions was considered preferable to water-based solutions. This was due to the hydrophobicity of allicin, which appeared more soluble in alcohol than in water. Ethanol also possessed hydroxyl groups of allicin stabilizers in the molecule [17].

In treating infected root canals, the root canal irrigants and medicaments used should have antimicrobial properties capable of disinfecting the root canal and additionally have no cytotoxic effect on periapical tissue and permanent tooth buds if pushed to the apex [10]. The addition of 2% CHX in the root canal treatment protocol could increase root canal disinfection [18]. CHX had a broad and effective antibacterial spectrum against Gram-positive, Gram-negative, fungal, and residual antibacterial effect [12,18]. However, by inhibiting protein synthesis and mitochondrial activities, CHX was highly cytotoxic to human periodontal ligament cells and harmed the fibroblast cells, where it also inhibited protein synthesis [19]. Our results implied that 25% garlic extract was effective in decreasing the viability of *E. faecalis* and was more effective than 2% CHX. Garlic extract, therefore, has great potential as an herbal root canal irrigant.

In this clinical study, we determined the efficacy of garlic extract as a root canal irrigant for primary teeth. Further studies are needed to maintain the stability of allicin in garlic extract to obtain a durable and optimal antibacterial effect.

## CONCLUSION

Our study demonstrates that garlic extract is effective in decreasing the viability of *E. faecalis* obtained from clinical isolates of nonvital primary root canals.

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**CONFLICT OF INTEREST**

The authors report no conflict of interest.

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