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## Effectiveness of *Lentinus edodes* Mushroom Extract on Eradication of *Enterococcus faecalis* Biofilm

Putri Lestari Anggiarini

*Postgraduate Program, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia*

Meiny F Amin

*Department of Conservative Dentistry, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia*

Juanita A Gunawan

*Department of Conservative Dentistry, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia*

Armelia Sari Widyarman

*Department Head of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia,*  
armeliasari@trisakti.ac.id

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### Cover Page Footnote

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**ORIGINAL ARTICLE**

**Effectiveness of *Lentinus edodes* Mushroom Extract on Eradication of *Enterococcus faecalis* Biofilm**

**Putri L. Anggiarini<sup>1</sup>, Meiny F. Amin<sup>2</sup>, Juanita A. Gunawan<sup>2</sup>, Armelia S. Widyarman<sup>3</sup>**

<sup>1</sup>Postgraduate Program, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia

<sup>2</sup>Department of Conservative Dentistry, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia

<sup>3</sup>Department Head of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta 11440, Indonesia

Correspondence e-mail to: armeliasari@trisakti.ac.id

**ABSTRACT**

Sodium hypochlorite is a commonly used irrigation solution in endodontic procedures, but it irritates tissues and has toxic effects. *Lentinus edodes* is a mushroom that has antibacterial properties. *Enterococcus faecalis* is an anaerobic bacterium that can cause root canal treatment failure. **Objective:** This study aimed to determine the effect of *L. edodes* extract on the eradication of *E. faecalis* biofilms. **Methods:** Phytochemical tests of *L. edodes* were performed to analyze alkaloids, steroids, triterpenoids, phenolics, tannins, flavonoids, and glycosides from this extract qualitatively. *E. faecalis* ATCC 29212 was cultured in brain heart infusion broth for 24 h at 37°C in an anaerobic atmosphere. Biofilm assay was performed to analyze the eradication of *E. faecalis* biofilm after treatment with *L. edodes* extract. The application times were 5, 15, and 30, and 10%, 20%, 40%, and 80% concentrations were used. Distilled water was used as a negative control, and NaOCl was used as a positive control. Data were statistically analyzed via one-way analysis of variance, where  $p < 0.05$  was set as the level of significance. **Results:** *L. edodes* mushroom extract was effective in eradicating *E. faecalis* biofilms in all concentrations and incubation times compared with the control ( $p < 0.05$ ). Significant differences were found between the application times of 5 and 15 min compared with 30 min ( $p < 0.05$ ). The most effective concentration in eradicating *E. faecalis* biofilms was 40% with an application time of 30 min. **Conclusion:** *L. edodes* mushroom extract proves its antibiofilm activity against *E. faecalis* biofilm. Further study is necessary to determine which substances have the most influence on the effectiveness of *L. edodes* extract in eradicating *E. faecalis* biofilm in vivo.

**Key words:** biofilm eradication, *Enterococcus faecalis*, *Lentinus edodes*, mushroom extract

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**INTRODUCTION**

Caries can develop over a long period of time on the entire surface of the tooth covered by biofilms.<sup>1</sup> Bacteria present in carious lesions can invade the pulp, causing reversible pulpitis. Pulp in irreversible pulpitis and pulp necrosis should be treated with root canals.<sup>2</sup> The most important factors that indicate the success of root canal treatment are good preparation, sterilization, and apical closure of obturation. The removal of necrotic pulp tissue and bacteria is an important factor. Imperfect obturation is a result of inadequate preparation and irrigation, leaving bacteria to grow in the root canal. Bacteria left in the root canal

form biofilms and cause further inflammatory reactions in the periapical region. If not treated properly, inflammation may worsen.<sup>3</sup>

Tooth root canal preparation supports the sterilization process and produces good filling; thus, optimal results are obtained. Preparation is carried out alternately with root canal irrigation solutions. Root canal irrigation is an important step in root canal treatment. It removes organic and inorganic necrotic tissue, kill microorganisms, and dissolve the smear layer during root canal preparation.<sup>2</sup> The ideal irrigation solution should be non-toxic, have antibacterial properties, function as a lubricant, have low surface tension, and be

able to dissolve the remaining necrotic pulp tissue and the smear layer formed during root canal preparation. Until now, there has not been an ideal irrigation solution that has all these properties. Existing irrigation solutions must be combined with other irrigation solutions, such as a combination of EDTA, sodium hypochlorite (NaOCl), and chlorhexidine.<sup>3</sup> NaOCl is a very commonly used solution for root canal irrigation. However, besides its being an irrigation solution, it also has a toxic effect on tissues: i.e., the higher its concentration, the worse its toxic effect. Some studies mention that NaOCl toxicity can cause pain, soft tissue edema, and paresthesias.<sup>4</sup>

Natural ingredients from traditional plants have been widely investigated as potentially better sources of antimicrobials. Mushrooms are alternative natural materials that have antibacterial effects. Fungi have bioactive metabolite ingredients that function as immunomodulators, protect the liver and cardiovascular system, and have antifibrotic, anti-inflammatory, antidiabetic, antiviral, antioxidant, and antitumor effects. Mushrooms can be a source of natural antibiotics.<sup>5</sup> Mushrooms contain peptides and proteins. Components found in fungi include sesquiterpenes, steroids, anthraquinones, benzoic acid derivatives, quinolones, oxalic acids, and phenols that function as antibacterial agents.<sup>6</sup>

*Lentinus edodes* is the most studied fungus. It appears to have broad antimicrobial effects on gram-negative and gram-positive bacteria. Extracts using chloroform, ethyl acetate, and water inhibit various gram-positive and gram-negative bacteria at concentrations below 50 mg/ml.<sup>7</sup> Ciric et al. examined the effectiveness of *L. edodes* mushroom extract to determine its antigingivitis effect.<sup>8</sup> Some literature has shown the effectiveness of *L. edodes* mushroom extract against *Streptococcus mutans* and *E. faecalis*.<sup>6,7</sup> *E. faecalis* is an anaerobic bacteria found in the root canal that can cause infection and inflammation. It is often associated with various forms of periradicular disease, as both primary (4%–44%) and persistent endodontic infections (24%–74%).<sup>9</sup> Some studies on the antibacterial effect of *L. edodes* mushroom extract on *E. faecalis* found that *L. edodes* has the ability to inhibit *E. faecalis*.<sup>6,10</sup> However, Carvalho stated that further investigation is required.<sup>11</sup> The present study was carried out to examine the effectiveness of the *L. edodes* mushroom extract in various concentrations and time applications in eradicating biofilm of *E. faecalis*. Previous studies focused on the effects of the extract on *E. faecalis*, not on its biofilm. Biofilm is more difficult to eliminate than planktonic bacterium. The results of this study can be used as baseline information on the use and selection of an irrigation solution in root canal treatment, which is more effective in reducing the prevalence of secondary infections after root canals caused by *E. faecalis*.

## METHODS

This experimental laboratory study was designed to determine the antibacterial effectiveness of fungal extracts of *L. edodes* on the eradication of *E. faecalis*. Concentrations of 10%, 20%, 40%, and 80% were applied for 5, 15, and 30 min. The study employed a post-treatment design with a control. This study was conducted at the Microbiology Center of Research and Education Laboratory of the Faculty of Dentistry, Trisakti University, Jakarta.

### *L. edodes* identification and phytochemical testing

The sample of *L. edodes* was taken from the Cibodas Bionic Farm, Puncak, West Java, Indonesia. The identification of the *L. edodes* mushroom plant was carried out at the Bogor Institute of Sciences, Bogor, Indonesia, and the phytochemical assay analysis was performed at the Indonesian Medicinal and Aromatic Crops Research Institute (BALITTRO) in Bogor, Indonesia.

### *L. edodes* extract preparation

A total of 200 g of dried *L. edodes* mushroom was immersed in 300 mL of water at 4°C overnight. The wet *L. edodes* material was crushed with a mixer, added to 1600 mL of chloroform, and stirred at 4°C overnight. Chloroform and insoluble material were separated by filtering or centrifugation. The chloroform extract was evaporated under low pressure to produce 876 mg of a light-yellow oily liquid. This insoluble material was then mixed with 1000 mL of water and stirred overnight at 4°C. The resulting suspension was then filtered or centrifuged to remove insoluble materials. After the pressure was reduced, the aqueous supernatant concentration was reduced to 200 mL. This concentration was then extracted by mixing with 200 mL of ethyl acetate three times. The organic layer was separated and evaporated at low pressure to produce a light-yellow ethyl acetate extract (114 mL). The aqueous extract was then freeze-dried and returned to a full water suspension. The solution obtained was diluted with sterile Distilled water to obtain concentrations of 10%, 20%, 40%, and 80%.

### Bacterial Culture and Biofilm Assay

*E. faecalis* ATCC 29212 was cultured in brain heart infusion broth for 24 h at 37°C in an anaerobic atmosphere. Subsequently, 200 µl of bacterial suspension ( $1 \times 10^7$  CFU/mL) was inserted into a 96-well plate and incubated for 24 h. The well plate was then rinsed with 200 µl phosphate buffered saline (PBS) and discarded. Each irrigation solution was put into the well plate up to 200 µl. The irrigation solution was incubated for 5, 15, and 30 min.<sup>12</sup> Moreover, the irrigation solution was discarded, and the well plate was rinsed again with 200 µl of PBS and discarded. Staining was then performed by adding a 200-µL solution of

**Table 1.** Phytochemical screening of the ethanol extracts of temu kunci

Plant constituents	Qualitative assay
Alkaloids	+
Saponins	-
Tannins	+
Phenolics	+
Flavonoids	+
Triterpenoids	+
Steroids	-
Glycosides	+

crystal violet into each well of the microtiter plate for 15 min and then rinsed with PBS. Ethanol acetone (200  $\mu$ L) was put into each microtiter well plate to check for biofilm formation. A microtiter plate reader was used to measure the optical density of violet crystals at 450 nm.<sup>13</sup> Sterile distilled water was used as a negative control and NaOCl as a positive control. The concentration of NaOCl used as the positive control was 5.25% as it is used as the golden choice for irrigation solution in root canal treatment. All treatments were done in three independent experiments in triplicates.

### Statistical Analysis

Data were analyzed using two-way analysis of variance and post-hoc tests to reveal significant differences between treatment groups. Data normality test was performed using the Kolmogorov–Smirnov test. Statistical significance was set at  $p < 0.05$ . Statistical calculations were performed using SPSS Statistics for Windows software version 20 (IBM Corp., Armonk, NY, USA).

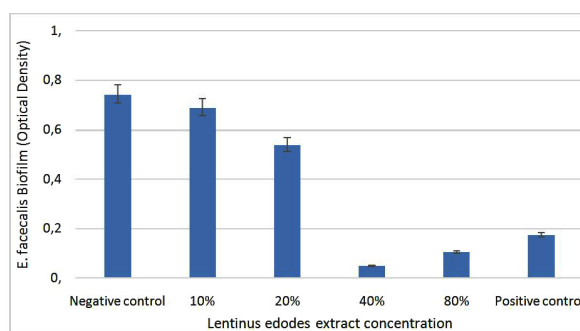
## RESULTS

The results of the phytochemical tests showed that the aqueous extract of *L. edodes* contained alkaloids, tannins, phenolics, flavonoids, triterpenoids, and glycosides (Table 1).

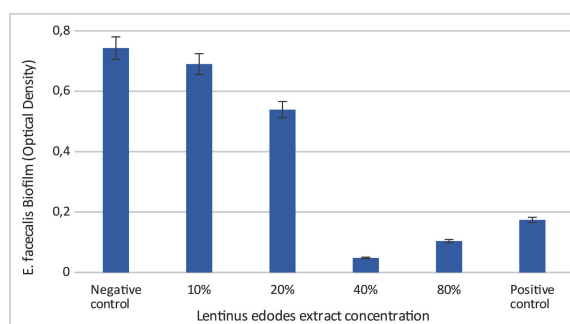
The results showed that *L. edodes* mushroom extract was effective in eradicating *E. faecalis* biofilms. This extract reduced the biofilm mass significantly in all concentrations and application times compared with the negative control ( $p < 0.05$ ). The average optical density and standard deviation of *E. faecalis* biofilms is shown in Figure 1-3. The most effective concentration is 40% applied for 30 mins, which eradicated 99.2% of biofilms compared with the negative control (without *L. edodes*).

## DISCUSSION

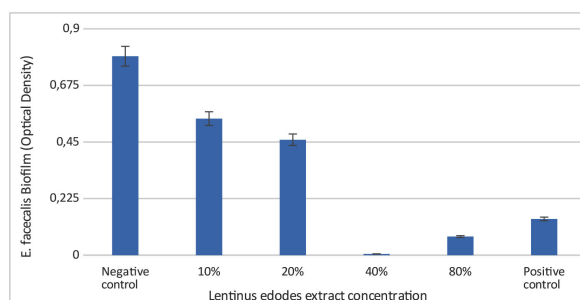
A comparative multiple test was performed using the post-hoc test to prove the significance of the



**Figure 1.** The biofilm assay of the *Lentinus edodes* extract against *E. faecalis* with 5 min of incubation time. The concentrations of *Lentinus edodes* extract applied were 80%, 60%, 40%, 20%, and 10%. Untreated biofilm wells were used as a negative control, while 5.25% sodium hypochlorite was used as a positive control.



**Figure 2.** The biofilm assay of the *Lentinus edodes* extract against *E. faecalis* with 15 mins of incubation time. The concentrations of *Lentinus edodes* extract applied were 80%, 60%, 40%, 20% and 10%. Untreated biofilm wells were used as a negative control, while 5.25% sodium hypochlorite was used as a positive control.



**Figure 3.** The biofilm assay of the *Lentinus edodes* extract against *E. faecalis* with 30 mins of incubation time. Concentrations of *Lentinus edodes* extract applied were 80%, 60%, 40%, 20% and 10%. Untreated biofilm wells were used as a negative control, while 5.25% sodium hypochlorite was used as a positive control.

differences between each ingredient. *L. edodes* extract with a concentration of 10%, 40%, and 80% showed significant differences compared with the negative control ( $p < 0.05$ ). When compared with 80% concentration, 40% did not give significant results, meaning that the antibacterial effect was not

significantly different between the two concentrations. Based on the application time, when the results of multiple applications, e.g., 5 and 15 mins, were compared with 30 mins, the results were significant ( $p < 0.05$ ).

*E. faecalis* reinfection and bacterial resistance are the most common causes of root canal treatment failure. In a study of 30 cases of endodontic failure, *E. faecalis* was found in 20 cases.<sup>14</sup> In primary endodontic cases, the prevalence of *E. faecalis* was 18%. Secondary endodontic cases had a higher prevalence of 67%.<sup>15</sup> *E. faecalis* can form biofilms in the root canal that are resistant to destruction. They are 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than other organisms that do not form biofilms. In addition, biofilms can reduce the quality of the covering of the root canal filling material.<sup>4</sup>

This study aimed to develop a natural ingredient as a safer irrigation solution for use in endodontic procedures. *L. edodes* was chosen because it is easy to find; it is a food ingredient that is used in various types of cooking. Hearst investigated the antimicrobial activity of the aqueous extract of the fungus *L. edodes* against *E. faecalis*. However, Carvalho showed the opposite results.<sup>10, 11</sup>

In this study, the content of *L. edodes* is consistent with those in the study by Alves and Macoris, who examined the phenolic content of the aqueous extract of the fungus *L. edodes*. They examined various phenolic components contained in the fungus *L. edodes*, namely, p-Hydroxybenzoic acid, protocatechuic acid, and cinnamic acid, which have antimicrobial power against *E. faecalis* by changing the permeability of cell membranes and damaging DNA.<sup>7, 16</sup>

Kuznetsov showed that a 5% concentration solution of *L. edodes* mushroom extract had antimicrobial power against *E. faecalis*.<sup>17</sup> Many studies have shown that some herbs, such as *Boesenbergia rotunda*, *Clitoria ternatea*, and *Myrmecodia pendans*, have been proven effective in inhibiting *E. faecalis*, *F. nucleatum*, *T. denticola*, and *P. gingivalis* as endodontic pathogen biofilms. These herbals are also known to have antibacterial substances, such as flavonoids, alkaloids, and tannins.<sup>18, 18, 20</sup>

Alkaloids can penetrate into the bacterial membrane and cause cytoplasmic leakage. Tannin is a type of compound that belongs to the polyphenol group. Tannin is able to deactivate bacterial adhesin, enzymes, and cell wall transport proteins and form complex compounds against extracellular proteins, which interfere with the integrity of the bacterial cell membrane. Its mechanism of action is by denaturing bacterial cell proteins and causing irreversible damage to cell membranes. Flavonoids form complexes with

cell walls and bind to adhesin. Triterpenoids can cause damage to bacterial cell membranes.<sup>21, 22, 23</sup>

The longer application time and higher concentration of *L. edodes* mushroom extract showed a significant difference when compared with the negative control, and the optical density value was lower. This demonstrates its effectiveness in eradicating *E. faecalis* biofilms. The results of this study are consistent with those of Hearst. However, the results are inconsistent with those of Carvalho et al., who concluded that *L. edodes* was not effective against *E. faecalis*. This difference may be attributed to differences in the part of the fungus studied, how it was extracted, and the incubation period.<sup>10, 11</sup>

## CONCLUSION

*L. edodes* mushroom extract was effective in eradicating *E. faecalis* biofilms. The most effective *L. edodes* extract was at a concentration of 40% applied for 30 min. This extract may be useful as antibiofilm agent. However, further research is needed on the effectiveness of *L. edodes* extract in vivo. Furthermore, it is necessary to study which substances have the most influence on the effectiveness of *L. edodes* extract in eradicating biofilms.

## CONFLICT OF INTEREST

The authors declare there are no conflicts of interest related to this study.

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