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Solution for Root Canal Treatment Failure: Comparison of Antibiofilm between Aloe Vera Extracts and Chitosan Shrimp Shells of the Formation Biofilm *Enterococcus Faecalis*

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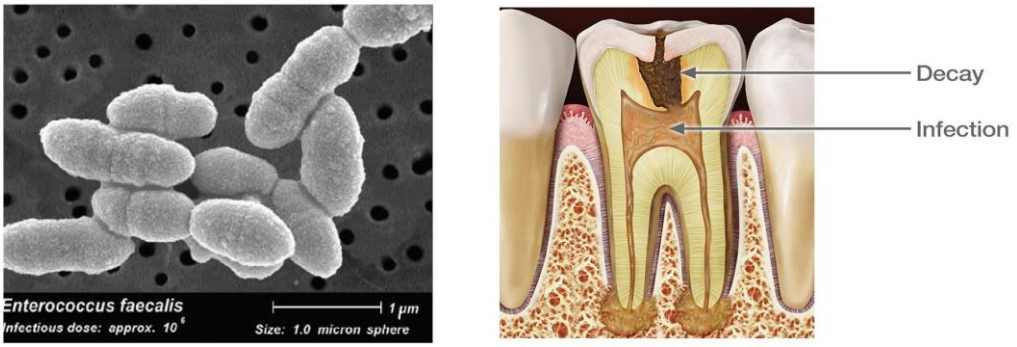
ABSTRACT

Enterococcus faecalis is a resistant bacterium in the root canal that can cause root canal treatment failure and is frequently found in root canal infections due to its ability to form biofilm. As a natural ingredient, aloe vera extract and chitosan can be used as an antibiofilm. This study aims to compare the antibiofilm properties of aloe vera extract and chitosan shrimp shells. The antibiofilm test was divided into two groups: groups I and II, tested 24 h and 72 h after biofilm formation by adding 0.1 mL of aloe vera suspension and 0.1 mL of chitosan suspension in 96 well plates containing *E. faecalis*. The aloe vera extract and chitosan concentrations used were 50%, 25%, 12.5%, and 6.25%. 0.1% crystal violet staining and OD values were measured using an ELISA reader. In the first group, aloe vera extracts at 25% concentration were more significant on positive control and had a percentage of biofilm inhibition of 93.77%, whereas chitosan at 25% concentration was most significant on positive control and had a percentage of biofilm inhibition of 90.59%. In the second group, aloe vera extracts of 12.5% concentration were most significant on positive control and had a percentage of biofilm inhibition of 90.68%, while chitosan at 25% concentration was most significant on positive control and had a percentage of biofilm inhibition of 88.37%. Aloe vera extract at a concentration of 12.5% can inhibit the growth of *Enterococcus faecalis* biofilms more effectively compared with that of the shrimp shell chitosan.

GRAPHICAL ABSTRACT

Solution For Root Canal Treatment Failure: Comparison of Antibiofilm Between Aloe Vera Extracts and Chitosan Shrimp Shells of The Formation Biofilm Enterococcus Faecalis



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Introduction

Enterococcus faecalis is a facultative anaerobic gram-positive bacterium with a prevalence of around 24% to 77% in a root canal; however, according to molecular studies, these bacteria have been present since pulp necrosis occurred. *E. faecalis* bacteria are found in approximately 4 to 40% of primary endodontic infections [1-6]. The prevalence of *E. faecalis* was found to be higher in the periradicular area. In primary endodontic infections, the failure rate of root canal treatment is nine times higher [7]. The majority of persistent apical periodontitis cases are caused by *E. faecalis* [8]. *E. faecalis* is frequently found in root canal infections because it has a high survival ability and grows in the micro-environment by colonizing to form a biofilm [9].

Biofilm is a collection of several multicellular microbes that form a colony and attach to a moist surface that is captured in extracellular polymeric substance (EPS) and attached to the wall surface of the root canal, providing a niche for bacteria [7, 10–13]. Root canal treatment is used to eliminate pathogenic bacteria, remove necrotic tissue, and aid in healing periapical wounds [14, 15]. Materials commonly used for conventional root canal irrigation have weaknesses because they are resistant to enterococci bacteria, thus requiring natural materials that can be developed as alternative materials for root canal irrigation that are less toxic, more biocompatible, and easily available. Natural materials that can be utilized for root canal irrigation are aloe vera extracts and chitosan shrimp shells [14, 16, 17].

Aloe vera is becoming increasingly popular due to its high concentration of tannins, polyphenols, amino acids, anthraquinones, minerals, vitamins, and salicylic acid. Aside from being antibacterial, aloe vera has other properties. The active substances that play a role are anthraquinone, saponin, and tannin [18–20]. Chitosan is a natural copolymer derived from shrimp shells composed of 2-acetamide-2-desoxi-D-glycopyranose linked by glycosidic bonds β. Chitosan has also been shown to inhibit, and damage matures biofilm [21]. Some previous research has demonstrated that chitosan can inhibit biofilms from *streptococcus viridans* at a concentration of 50%, and aloe vera extracts have the ability to inhibit the growth of *E. faecalis* bacteria [17, 19]. The effectiveness of aloe vera extracts and chitosan to inhibit biofilm can be utilized to determine which of them should be used as root canal irrigation. Based on the explanation above, it is necessary to conduct research on the antibiofilm comparison of aloe vera extracts and chitosan shrimp shells in the formation of *E. faecalis* biofilm using ELISA.

Research Methodology

This research is an in-vitro laboratory experimental study with Post-test Only Group Design research using bacterial samples formed into *E. faecalis* biofilm ATCC 29212. This research was conducted at the Microbiology Laboratory of Universitas Brawijaya in November 2020.

The samples used were aloe vera obtained from the UPT Laboratory of Herbal Materia Medica Batu, Malang, which was extracted using 96% ethanol solvent by maceration method, and chitosan powder obtained from Bogor Agricultural Institute (IPB), which were then diluted with 1% acetic acid. The research instruments used were pipettes, test tubes, ELISA kits, and an ELISA reader (Biogear Light Motion Microplate Reader).

Biofilm formation

E. faecalis ATCC 29212 was used in this research. This strain has the ability to form biofilm. Inoculums for the biofilm assays were prepared overnight (24 h) in *tryptic soy broth* (TSB) and incubated at 37 °C for group I, and 3x24 h (72 h) in *tryptic soy broth* (TSB) and incubated at 37 °C for group II.

Antibiofilm assay

In group I, biofilm assays was done using 96-well plates filled with 200 µL of TSB (Trypticase Soy Broth) and 1% (w/v) glucose with inoculum biofilm for 24 h with 0.1 mL of aloe vera, and other wells were filled with 200 µL of TSB (Trypticase Soy Broth) and 1% (w/v) glucose with inoculum biofilm for 24 h with 0.1 mL of chitosan suspension each concentration and different well.

In group II, biofilm assays were done using 96-well plates filled with 200 µL of TSB (Trypticase

Soy Broth) and 1% (w/v) glucose with inoculum biofilm for 72 h with 0.1 mL of aloe vera, and other wells were filled with 200 µL of TSB (Trypticase Soy Broth) and 1% (w/v) glucose with inoculum biofilm for 72 h with 0,1 mL of chitosan suspension each concentration and different well.

Aloe vera extract and chitosan used concentrations of 50%, 25%, 12,5%, and 6,25% with 8 replications. After 24 h of incubation at 37 °C, the contents of each well were discarded and washed with phosphate buffer saline (PBS) 3 times to remove planktonic bacteria before being dried. Biofilm growth was assessed using biomass, and the inhibition percentage was measured using optical density (OD).

Biomass production inhibition was performed using the crystal violet staining assay (Costa el, 2017). Staining was performed with 0.1% crystal violet solution as much as 0.2 mL and was incubated at 37 °C for 15 min. The remaining staining liquid was washed with distilled water several times until there was no color in the rinse water, then dried, and 0.1 mL 100% DMSO was added. Biofilm growth was measured by the OD 660 nm using a microplate reader (ELISA reader, Biogear Light Motion Microplate Reader). Bacteria *E. faecalis* culture medium in TSB supplemented with glucose was used as a positive control. All assays were completed, and the results were expressed as a percentage of biomass inhibition (OD_{sample} is the optical density measured for each sample and $OD_{positive\ control}$ is the optical density of the positive control).

The formula for the percentage of biofilm growth inhibition is [22–24]: $100- (OD_{sample}/OD_{positive\ control}) \times 100$.

Data Analysis

Data analysis was performed using SPSS 16.00 version.

Difference Tests

The results of antibiofilm of aloe extract and chitosan were collected and then presented in a table and analyzed using the data normality test with Kolmogorov-Smirnov, Homogeneity Test with Levene's test, and One-way ANOVA coupled with LSD's *post hoc* test and Tamhane's *post hoc* test to determine the differences between the results observed with considered significant for p-values < 0.05.

Results and discussion

This research aims to investigate the antibiofilm effects of aloe vera extract and shrimp shell chitosan on the formation of *E. faecalis* biofilm. Based on the reading of the OD (Optical Density) function, which was used to see the turbidity of biofilms using ELISA reader, it was found that biofilms formed from aloe vera extract and chitosan from the four concentrations had lower OD values after 24 h (group I) and 72 h (group II). The positive OD value of the control group (+) indicates that aloe extract and chitosan can inhibit biofilms.

Table 1: Group I (Results of mean OD values of *E. faecalis* biofilm with shrimp shell chitosan)

Concentration group	Samples	Mean OD	Standard Deviation
50%	8	0,2026	0,02153
25%	8	0,1499	0,02262
12,5%	8	0,4388	0,09383
6.25%	8	0,6981	0,06047
Positive Control	8	1,5941	0,22245

Table 2: Group II (Results of mean OD values of *E. faecalis* biofilm with shrimp shell chitosan)

Concentration group	Samples	Mean OD	Standard Deviation
50%	8	0,2262	0,01205
25%	8	0,1341	0,01737
12,5%	8	0,4909	0,05337
6.25%	8	0,3388	0,06175
Positive Control	8	1,1533	0,14896

Table 3: Group I (Results of mean OD values of *E. faecalis* biofilm with aloe vera extracts)

Concentration group	Samples	Mean OD	Standard Deviation
50%	8	0,1746	0,03228
25%	8	0,0993	0,01557
12,5%	8	0,1266	0,03546
6.25%	8	0,1008	0,01522
Positive Control	8	1,5941	0,22245

Table 4: Group II (Results of mean OD values of *E. faecalis* biofilm with aloe vera extracts)

Concentration group	Samples	Mean OD	Standard Deviation
50%	8	0,1484	0,02192
25%	8	0,1079	0,01261
12,5%	8	0,1074	0,01555
6.25%	8	0,1813	0,03515
Positive Control	8	1,1533	0,14896

Table 5: Group I (Percentage inhibition of aloe vera extracts against *E. faecalis* biofilms)

Concentration Group	Mean OD	% Biofilm growth inhibition
50%	0,1746	89,04%
25%	0,0993	93,77%
12,5%	0,1266	92,05%
6,25%	0,1008	93,67%
Positive Control	1,5941	0%

Table 6: Group II (Percentage inhibition of aloe vera extracts against *E. faecalis* biofilms)

Concentration Group	Mean OD	% Biofilm growth inhibition
50%	0,1484	87,13%
25%	0,1079	90,64%
12,5%	0,1074	90,68%
6,25%	0,1813	84,27%
Positive Control	1,1533	0%

Table 7: Group I (Percentage inhibition of shrimp shells chitosan against *E. faecalis* biofilms)

Concentration Group	Mean OD	% Biofilm growth inhibition
50%	0,2026	87,29%
25%	0,1499	90,59%
12,5%	0,4388	72,47%
6,25%	0,6981	56,20%
Positive Control	1,5941	0%

Table 8: Group II (Percentage inhibition of shrimp shells chitosan against *E. faecalis* biofilms)

Concentration	Mean OD	% Biofilm growth inhibition
50%	0,2262	80,38%
25%	0,1341	88,37%
12,5%	0,4909	57,43%
6,25%	0,3388	70,62%
Positive Control	1,1533	0%

Based on the data in the table above, it can be seen in Tables 5 and 7 that at a concentration of 25%, aloe extract and chitosan can both equally inhibit *E. faecalis* biofilm. Tables 6 and 8 show that at 12.5% concentration of aloe extract can inhibit biofilm formation after 72 h, whereas a 25% concentration of chitosan can inhibit *E.*

faecalis biofilm formation. The barrier of biofilm of aloe extract and chitosan both reached 90%.

The results of data analysis using the Kolmogorov Smirnov test [25, 26] showed that the data were normally distributed p> 0.05. However, the homogeneity test using the Levene's test [27, 28] revealed that the data were not homogenous (p<0.05). The One-Way ANOVA test was performed to examine the differences between treatment groups and yielded p = 0,000 (p <0.05) that there were significant differences with positive controls. The Post Hoc Multiple Comparison (Tamhane) test was then performed to find data groups with significant differences in the value of OD biofilms.

Based on the Post Hoc Multiple Comparison (Tamhane) test results, chitosan shell shrimp with concentrations of 50%, 25%, 12.5%, and 6.25% significant towards positive control (p = 0,000) in group I, and aloe extract with concentrations of 50%, 25%, 12.5%, and 6.25% were significant for positive control (p = 0,000).

In group I, chitosan shell shrimp at a concentration of 25% was the most significant in the positive control, while aloe extract at a concentration of 25% was the most significant in the positive control. Based on the percentage of inhibition calculated by the formula above, the chitosan shell at a concentration of 25% has the highest percentage of inhibition (93.77%). In comparison, aloe extract at a concentration of 25% has the greatest percentage of inhibition (90.59%).

In group II, chitosan shell shrimp at concentrations of 50%, 25%, 12.5% and 6.25% were significant to positive control (p = 0,000), and aloe extract with concentrations of 50%, 25%, 12.5% and 6, 25% was significant to positive control (p = 0,000). In group II, shrimp shell chitosan at a concentration of 25% showed the most significant influence on positive control, while aloe extract at a concentration of 12.5% also showed the most significant influence on positive control. Based on the percentage of inhibition calculated using the formula above, chitosan shell at a concentration of 25% has the highest inhibition percentage (88.87%), whereas aloe extract at a concentration of 12.5% has the highest inhibition percentage (90.68%).

This research aims to compare the antibiofilm properties of aloe vera extracts and shrimp shell chitosan on the formation of *E. faecalis* biofilms. *E. faecalis* bacteria were used in this research because this bacterium was frequently found resistant in the root canal, even after it had been cleaned with root canal irrigation material, and this is what caused the failure of root canal treatment [29, 30].

In vitro research on *E. faecalis* biofilm was conducted using the microtiter plate assay method and an ELISA reader for Optical Density readings. Aloe extracts were obtained from the maceration process with 96% ethanol solvent, and chitosan solution was obtained by dissolving it in 1% acetic acid.

The results show that all of the OD values in the biofilm group were lower than the OD values in the control group. The lower the OD value obtained, the higher the percentage of biofilm inhibition. At 24 h after biofilm formation, aloe extract could inhibit biofilm at a concentration of 25%, with biofilm inhibition reaching 93.77%, while chitosan could inhibit biofilm at a concentration of 25%, with biofilm inhibition reaching 90.59%. At 72 h after the biofilm extract formation, it could inhibit biofilm at a concentration of 12.5%, with biofilm inhibition reaching 90.68%, while the chitosan could inhibit the biofilm at a concentration of 25%, with biofilm inhibition reaching 90.59%.

The time difference between 24 h and 72 h after the formation of *E. faecalis* biofilms shows that bacteria go through a lag phase, exponential growth phase, stationary growth phase, and decline growth phase. In theory, the *E. faecalis* bacterium experiences the highest exponential growth phase at 14 h, followed by an exponential growth phase during the first 24 h. According to the existing theories, bacteria will adapt to a bad environment by forming biofilms, so that the OD value is still high after 72 h compared to the first 24 h [29].

The Tamhane test revealed that in the first 24 h group, the 25% concentration of aloe vera had a significant difference compared to other

concentrations, and the biofilm inhibition reached 93.77%, the highest among the concentrations compared to chitosan. In the first 72 h group, the concentration of 12.5% of Aloe vera extract had a significant difference compared to other concentrations, and the biofilm inhibition reached 90.59%, the highest among the concentrations compared to chitosan. This demonstrates that aloe extract at a concentration of 12.5% can inhibit the growth of *E. faecalis* biofilm more effectively than shrimp shell chitosan.

The inhibition of aloe vera biofilm is better than chitosan because chitosan has a saturation point in damaging the mature biofilm, and it has to bind with new theatrical acid that can damage the cell membrane and cause bacterial lysis [15]. Anthraquinone, saponin, and tannin are compounds contained in aloe vera extracts. Tannin compounds in aloe vera can denaturize proteins and bind iron ions needed by bacteria to maintain the biofilm matrix, resulting in a decrease in bacterial viscosity and a decrease in the biofilm matrix bond [31, 32].

Conclusions

Endodontic treatment failure may occur due to different causes such as persistence of bacteria, root canals that are poorly cleaned and obturated, improper coronal seal (leakage), and untreated canals (missed canals). The main reason for endodontic failure is the presence of some species of bacteria inside the root canal system such as Enterococcus (E.) faecalis. Those bacteria are more resistant to disinfection agents, causing a persistent intra-radicular or extra-radicular infection. This study aims to compare the antibiofilm properties of aloe vera extract and chitosan shrimp shells. In conclusion, our research discovered that aloe extracts at a concentration of 12.5% could inhibit the growth of *Enterococcus faecalis* biofilms more effectively than shrimp shell chitosan.

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Authors' contributions

All authors contributed toward data analysis, drafting and revising the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

We have no conflicts of interest to disclose.

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