Anti-microbial efficacy of Allium sativum extract against Enterococcus faecalis biofilm and its penetration into the root dentin: An in vitro study

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ABSTRACT

Introduction: Sodium hypochlorite (NaOCl) has long been the most preferred root canal irrigant in endodontic treatment, but besides being an effective anti-microbial agent, it is highly cytotoxic. Thus, a search for an alternative herbal irrigant which would be more biocompatible but equally effective led to this study.

Aim: To assess the anti-microbial efficacy of garlic extract (GE) against Enterococcus faecalis biofilm and its ability to penetrate into root dentin.

Materials and Methods: E. faecalis was cultured and treated with the test agents - normal saline, 5.25% of NaOCl, and the three different concentrations of GE (10%, 40%, and 70%). The experiment was done in four groups namely, 24-h Co-treatment group, 24-h biofilm treatment group, 1-week biofilm group, and 3-week biofilm group. These groups were subjected to microbial viability assay and fluorescence microscopic analysis. The most effective concentration of garlic (70%) was further tested and compared with 5.25% NaOCl for its dentin penetration property using 0.2% alizarin red under a fluorescence microscope.

Results: The findings revealed that GE was able to disrupt as well as prevent the formation of biofilm produced by E. faecalis. All the concentrations of GE displayed considerable anti-microbial efficacy where 70% concentration was most effective and exhibited similar anti-microbial efficacy as 5.25% NaOCl. In terms of dentin penetration, no significant difference was found between GE and NaOCl.

Conclusion: The results indicate that GE has a potential to serve as an alternative herbal root canal irrigant being an effective and biocompatible anti-microbial agent with good dentinal penetration property.

Key words: Biofilm, dentine penetration, Enterococcus faecalis, fluorescence microscopy, garlic extract

There are many requisites for endodontic treatment to succeed: Adequate cleansing and proper shaping of the canal followed by satisfactory root canal filling. The recommended procedures for the biomechanical preparation of root canals are well-established. Existing literature is replete with studies regarding various techniques of canal preparation, from establishing the correct working length to the appropriate shaping of canals, such as serial preparation and crown-down techniques.1,2

One avenue of investigation that also deserves much attention is the search for endodontic materials that will contribute to further increase the success of root canal treatment. To this end, many researchers have studied the

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various types of newer files,[3,4] as well as different types of gutta-percha, sealers and cements. Studies regarding root canal irrigants; however, are fewer, which may be due to the fact that sodium hypochlorite (NaOCl) has long been the gold standard and has been proven to be effective in disinfecting the canal as well as dissolving any remnants of pulp tissue.[5-7]

Despite the success of NaOCl as a root canal irrigant, it is still worth looking into possible alternatives because it shows to be high toxicity, resulting in necrotic effects when it comes in contact with normal tissues.[8-10]

Consequently, the search for alternative products such as natural phytochemicals isolated from plants used in traditional medicine would be reasonable.[11]

One such plant product containing natural phytochemicals is garlic. Garlic (Allium sativum) is one of the most extensively investigated medicinal plants in use since ancient times due to its anti-bacterial, anti-fungal, and anti-viral properties.[12] Its extract has been shown to have a wide spectrum inhibitory effect on the growth of various Gram-positive and Gram-negative bacteria.[13-15]

In this study, the authors investigated the efficacy of garlic (A. sativum) extract to reduce the number of viable Enterococcus faecalis, to prevent the formation of biofilm produced by E. faecalis and its ability to penetrate an existing biofilm, along with its property to penetrate into the dentinal tubules.

MATERIALS AND METHODS

Preparation of garlic extract
Fresh natural garlic (A. sativum) was obtained from the Bureau of plant industry (Department of Agriculture), Manila. It was then blended in a sterilized mortar and pressed with gauze. This extract was centrifuged at 12,000 rpm for 10 min and then filtered with a 0.45-mm filter to obtain raw garlic extract (GE) and stored at −20°C until use.

Culture of Enterococcus faecalis and treatment with agents
E. Faecalis strain (ATCC 47077) was inoculated in brain heart infusion broth and incubated overnight at 37°C in a shaker incubator. A standard E. faecalis bacterial suspension was prepared using McFarland 0.5 (1.5 × 10⁶ CFU/ml). E. faecalis bacterial suspension (100 µL) was dispensed into four sterile flat-bottomed 96-well polystyrene microtiter plates. In the first plate, designated as the "co-treatment" group, raw GE was immediately added to E. faecalis (100 µL) suspension to obtain 10%, 40%, and 70% concentrations and then incubated at 37°C for 24 h. For the second, third, and fourth plates, E. faecalis suspension was incubated at 37°C for 24 h, 1-week, and 3 weeks, respectively, to allow for the growth and formation of biofilm. These were designated as the “24-h,” “1-week,” and “3-week” groups. The medium was replaced regularly to replenish the nutrients. After the allotted time, the three remaining groups (24-h, 1-week, and 3-week groups) were each treated with 10%, 40%, and 70%, respectively, GE in the same manner. For comparison, controls were prepared for all the plates as follows: Positive control (E. faecalis + normal saline [NS]), negative control (E. faecalis + 5.25% NaOCl). A total of three wells were inoculated per concentration and per agent; hence, all the samples were processed in triplicates. For all the experimental groups, the biofilms were exposed to the agents for 10 min after which, the agents were carefully removed with a micropipette. 100 µL sterile deionized distilled water was then added, after which the microbial viability assay and biofilm staining procedures were performed.

To determine the amount of viable bacteria 10 µL of Presto Blue cell viability reagent (Invitrogen, USA) was added to all the wells in the microtiter plates. The plates were incubated at 37°C for 30 min and then read at 570 nm using microplate reader (Biotek, USA) to determine the colorimetric data optical density (O.D.). The inhibition index was then calculated using the following formula: Inhibition index (%) = 100 − ([O.D sample/O.D control] × 100).

Biofilm formation was confirmed by SYPRO® Ruby Biofilm Matrix stain (Invitrogen, USA) and observed under the fluorescence microscope (Applied Spectral Imaging, Israel). 10 µL of staining solution was added gently onto the biofilm. The samples were incubated for up to 30 min at room temperature protected from light. They were then rinsed gently with filter sterilized water to remove all the stain and viewed under the fluorescence microscope. All the tests were performed according to the manufacturer's instructions.

Assessment of dentin penetration
The approval from Institutional Review Board was taken before conducting the study (Ref: 0013/E/O/10/11). To assess whether GE could penetrate into the dentinal tubules, the protocol described by Paragliola and Franco,[16] with slight modifications, was followed. Briefly, 10 recently extracted human single canal teeth were decoronized to standardize the lengths. The working length was established by inserting a size 10 K-type file into each canal until it was seen through the apical foramen then retracting it by 0.5 mm. The canals were shaped with nickel-titanium rotary instruments (Protaper, Dentsply Maillefer, Switzerland) following the usual protocol, with size F3 as the last file used at the working length.

The specimens were divided into two groups (five in each). The first group was irrigated with 3 mL of 5.25% NaOCl labeled with 0.2% alizarin red while the second group was irrigated with 3 mL of 70% GE labeled with 0.2% alizarin red
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at each instrument change with a 30-G needle at 5 mm from the working length. Smear layer removal was achieved after irrigation in both groups with 3 mL of 17% EDTA (Pulpdent, USA) followed by 3 mL of sterile saline. A final rinse of each canal was performed by using 5 mL of alizarin labeled with 5.25% of NaOCl and 70% GE in each group, respectively.

After drying the canals, each specimen from the both groups was horizontally cut into three 1-mm thick sections at 1, 3, and 5 mm from the apex, designated as Section 1, Section 3, and Section 5, respectively. The sections were then bonded onto glass slides and were ground with wet silicon carbide papers to approximately a 40 µm thickness. The slides were examined with a fluorescence light microscope at ×100 with a wavelength of 540 to 570 nm. Images from all the specimens were evaluated by three blinded operators. The following set of scores was used to assess the penetration of the irrigant solution into the dentinal tubules [Figure 1]: “1”- minor (penetration in <50% of dentinal tubules) and “2”- major (penetration in ≥50% of the dentinal tubules).

Data obtained were analyzed using ANOVA followed by mean comparison to compare the biofilm formation and disruption between the control and the experimental groups and among the experimental groups using SPSS Version 16 (SPSS for Windows, Chicago, SPSS Inc.). Chi-square with the two-sided Likelihood ratio test and one-sided Fisher’s exact test were done to check the dentin penetration. The level of significance was established as \( P < 0.05 \) for the statistical tests.

RESULTS

Viable microorganism count

The mean values of the data gathered for the viable microorganism count of the five treatment groups at all the time intervals revealed that negative control, 40% and 70% GE displayed considerable inhibition of the bacterial growth. 70% GE and negative control displayed similar results with no significant difference between them (\( P = 1.0 \)) while the effect of 10% GE was similar to the positive control [Figure 2].

Evidence of biofilm formation for the control groups

Biofilm formation, as assessed by the modified SYPRO Ruby stain was expressed in relative fluorescence units (RFUs) read at 620 nm. A high RFU value is interpreted as the presence of biofilm formation and conversely, a low RFU value means that there is little or no biofilm formation. The positive control (E. faecalis + NS) of 3-week biofilm group showed a thick, dense, and clustered biofilm formation as noted by the very high fluorescence values when compared to the positive control of other biofilm groups, while the negative control (5.25% NaOCl + E. faecalis) displayed no biofilm formation with low RFU values [Table 1].

Evidence of disruption of biofilm formation for the treatment groups

For all the groups, it was observed that 10% GE exhibited slight disruption in biofilm formation as compared to the positive control. 40% GE showed a substantial disruption of biofilm when compared to the positive control (\( P < 0.05 \)). Whereas, no biofilm formation was observed with 70% GE as evident from the significantly smaller RFU value as compared to the positive control (\( P < 0.01 \)). No significant difference was found between 70% GE and negative control groups (\( P = 0.939 \)) [Figure 3].

Results for the fluorescence values of the treatment groups are summarized in Table 1. Taken all together, the results indicated that there was increasing disruption of E. faecalis biofilm formation as the concentration of the GE was increased. It can be seen from Figure 4 that NaOCl and 70% GE had the lowest fluorescence values while the positive control (E. faecalis + NS) had the highest fluorescence value.

Penetration of garlic extract into the dentinal tubules

Table 2 shows the cross-tabulation displaying major and minor dentin penetration of both the treatment groups in Sections 1, 3, and 5. The result revealed that GE had a slightly higher dentin penetration than 5.25% of NaOCl but the difference was not significant. This was confirmed by both the two-sided Likelihood ratio test (\( P = 0.063 \)) and one-sided Fisher’s exact test (\( P = 0.070 \)), which showed that there was no significant difference in dentin penetration for the specimens irrigated with GE and NaOCl.

![Figure 1: Fluorescence microscopic images of dentine penetration by alizarin red dye](image1)

![Figure 2: Comparison of the mean values of the optical density representing the amount of viable bacteria in the treatment groups (**\( P < 0.01 \), *\( P < 0.05 \))](image2)
DISCUSSION

Several investigations have studied the anti-microbial effects of GE; however, there is very limited information about its anti-microbial efficacy against \textit{E. faecalis} biofilm formed in root canals and its penetration into the root dentin.

In the present study, it was found that 40% and 70% concentration of GE has significant anti-microbial activity against \textit{E. faecalis} biofilm, with 70% concentration being as potent as 5.25% of NaOCl in reducing the number of \textit{E. faecalis} as well as in preventing and removing the bacterial biofilm. This is in line with a study conducted by Borhan-Mojabi \textit{et al.}, in 2012, where 40% and 70% concentration of GE were found to be effective in significantly reducing the total salivary microbial population.\cite{17} Another study done by Zakaria 2004, demonstrated that the aqueous GE was effective against an array of Gram-positive and Gram-negative pathogens.\cite{18} With regards to its use in endodontics, a study done by Abu and Sawsan in 2001, suggested that fresh minced garlic solution can be useful as an intracanal medication as it inhibited the growth of \textit{E. faecalis} and \textit{Pseudomonas aeruginosa}, \textit{α}-hemolytic \textit{Streptococci}, and \textit{Streptococci pyogenes}.\cite{19}

The minimum inhibitory concentration (MIC) of GE on \textit{E. faecalis} growth as determined by Lee \textit{et al.}, is 128 mg/ml (12.8%).\cite{20} This explains the limited effect of 10% concentration of GE (sub-MIC) in reducing the number of viable \textit{E. faecalis} as well as its biofilm.

It is worth mentioning that careful consideration must be taken to preserve the effectivity of the GE in its method of preparation. In this study, garlic bulbs were crushed and the extracts taken and filtered without subjecting them to any

Table 1: Fluorescence values (expressed in RFU*) of \textit{E. faecalis} biofilms as assessed by the modified SYPRO Ruby staining protocol for the treatment groups

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Co-treatment</th>
<th>24-h biofilm</th>
<th>1-week biofilm</th>
<th>3-week biofilm</th>
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</thead>
<tbody>
<tr>
<td>\textit{E. faecalis} + 10% GE</td>
<td>405,552</td>
<td>541,401</td>
<td>552,728</td>
<td>581,284</td>
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<tr>
<td>\textit{E. faecalis} + 40% GE</td>
<td>317,979</td>
<td>335,786</td>
<td>357,719</td>
<td>381,354</td>
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<td>\textit{E. faecalis} + 70% GE</td>
<td>157</td>
<td>272</td>
<td>1691</td>
<td>13,289</td>
</tr>
<tr>
<td>Positive control</td>
<td>611,071</td>
<td>661,519</td>
<td>695,977</td>
<td>770,836</td>
</tr>
<tr>
<td>Negative control</td>
<td>117.5</td>
<td>308</td>
<td>1727</td>
<td>3345</td>
</tr>
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</table>

*RFU=Relative fluorescence units (high RFU=Thick biofilm formation; low RFU=Little or no biofilm formation), GE=Garlic extract, \textit{E. faecalis}=\textit{Enterococcus faecalis}, NS=Normal saline

![Figure 3: Fluorescence microscopic images showing \textit{Enterococcus faecalis} biofilm in all the groups. (a-e) Represents co-treatment group; (f-j) represents 24 h biofilm group; (k-o) represents 1-week biofilm group, and (p-t) represents 3-week biofilm group](http://www.ijdr.in)
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exposure to high temperature during the entire procedure to preserve allicin which is the most important anti-microbial component of garlic and to eliminate the possibility of inactivation of any of its important components. This effective procedure of preserving allicin from being deactivated has been confirmed by a study conducted by Chavan *et al.* in 2010 which reported that many therapeutic properties in garlic are destroyed when subjected to heat.[21]

It has been recognized that allicin, the unstable compound formed by the enzymatic action of alliinase is inactivated at a temperatures above 65°C.[22]

One of the important properties of NaOCl as a potent root canal irrigant is its ability to penetrate into the dentinal tubules. Several studies have been done to check and analyze the dentin penetration ability of the different concentrations of NaOCl. According to a study conducted by Zou *et al.* in 2008 which reported that many therapeutic properties in garlic are destroyed when subjected to heat.[21] In the present study, it was seen that 70% concentration of GE exhibited good dentinal penetration similar to that of 5.25% NaOCl.

The present findings suggest that based on the demonstrated capabilities, GE may provide benefits as an herbal root canal irrigant in preventing *E. faecalis* biofilm formation inside the root canals and may effectively penetrate into the root dentin. Lack of sufficient in vivo studies; however, prohibits its clinical practice recommendation at the present time. Further clinical investigations for standardization and preparation of the irrigant containing this anti-microbial agent for the prevention of bacterial biofilm formation in the root canal is proposed to confirm the efficacy of GE for their treatment. Further studies may also look into the possibilities of masking the strong odor of garlic extract so as to be of practical use in the clinical setting.

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Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES


![Table 2: Cross-tabulation of dentin penetration by treatment](http://www.ijdr.in)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Garlic section 1</th>
<th>Garlic section 3</th>
<th>Garlic section 5</th>
<th>NaOCl section 1</th>
<th>NaOCl section 3</th>
<th>NaOCl section 5</th>
</tr>
</thead>
<tbody>
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<td>Dentin penetration</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td>Expected count</td>
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<td>2.2</td>
<td>2.2</td>
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<td>2.2</td>
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<tr>
<td>2 Count</td>
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<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Expected count</td>
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<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

![Figure 4: Mean values of fluorescence classified according to the treatment. Positive control (*Enterococcus faecalis* + normal saline); negative control (*Enterococcus faecalis* + sodium hypochlorite); 10% (*Enterococcus faecalis* + 10% garlic extract); 40% (*Enterococcus faecalis* + 40% garlic extract); 70% (*Enterococcus faecalis* + 70% garlic extract)](http://www.ijdr.in)
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