Antimicrobial effect of phytic acid on Enterococcus faecalis

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Abstract

Objectives: One of the properties of an ideal root canal irrigant is the ability to eradicate Enterococcus faecalis which is one of the most resistant microorganisms encountered in persistent peri-radicular lesions. The aim of this study was to test the in vitro antibacterial effectiveness of a naturally occurring agent called phytic acid (IP6) against E. faecalis and compare it to the antibacterial activities of clinically used irrigants: sodium hypochlorite (NaOCl), ethylenediaminetetraacetic (EDTA), phosphoric acid (PA) and chlorhexidine (CHX).

Design: The antimicrobial activities of 5% IP6, 5% NaOCl, 18% EDTA, 37% PA and 2% CHX against E. faecalis were determined using disk diffusion test. Minimum inhibitory concentration (MIC) was calculated by broth macrodilution method. The minimal bactericidal concentration (MBC) was determined for the used agents by culturing the clear broth of MIC tests.

Results: The results of agar diffusion test showed statistically significant differences between the groups. PA showed a larger zone when compared to other tested materials (p< 0.05). There was no statistical significant difference between NaOCl, EDTA and CHX (p=0.098). IP6 showed the smallest zone of inhibition when compared to all groups (p< 0.05). The recorded MIC and MBC values for IP6 were 0.156% and 0.625% respectively. The MIC and MBC values for PA were 0.578% and 4.6% and for NaOCl 0.093% and 0.375%, respectively. EDTA MIC value was 0.14 % but it showed no bactericidal activity. CHX was excluded from MIC test as immediate precipitation and turbidity occurred after mixing CHX with Mueller Hinton Broth.

Conclusions: Within the limitation of this study and despite that IP6 showed the smallest zone of inhibition in agar diffusion test, the results of MIC and MBC indicated that IP6 exhibits in vitro antibacterial effect against E. faecalis at low concentrations.

Keywords: Phytic acid, Enterococcus faecalis, Antibacterial effect, Roots canal irrigant.
Introduction

Root canal treatment is an integral part of common dental practice. Despite the high success rate of this treatment, failure is not uncommon. Many etiologica factors can lead to this failure; however, intra-radicular or secondary infections are the major causes [1]. Bacteria play an important role in the failure of root canal treated-teeth, hence the need to use antimicrobial agents during the treatment. Enterococcus faecalis, a gram positive facultative anaerobic bacterium, is the most commonly microorganism associated with endodontic failure and persistent infections [2]. This bacterium is known for its ability to resist many of the clinically used antibacterial agents [3]. Sodium hypochlorite (NaOCl) is the main irrigant used in root canal treatment [4], despite its many advantages it has several drawbacks which are mainly related to its toxicity on vital tissues and corrosion of metals [5]. Other used irrigants are ethylenediaminetetraacetic acid (EDTA) and chlorhexidine (CHX). The former is mainly used as a chelating agent to remove the smear layer [6, 7] and the latter is used for its antimicrobial effectiveness and substantively to denitin [8, 9]. However, these two agents are synthetic materials that may negatively affect the vitality of healthy tissue around the teeth, thus affecting the prognosis of post-treatment recovery or even result in chronic post-operative pain [10-13]. Phosphoric acid (PA) has been also suggested as root canal irrigant for the purpose of removing the smear layer due to the ability of removing the latter [14] and its high antimicrobial effectiveness; however, its toxicity was higher when compared to other smear layer removal agents [15].

Phytic acid (IP6) is naturally occurring compounds that has many medical applications and proved to be safe for human use [16]. IP6 is the major storage form of phosphorus in plant seeds and barns [17]. This material can be extracted with low cost from rice bran [16]. It has negative charge, making it effective in chelating multivalent metal ions, especially magnesium, calcium, and iron [18], which results in poor bioavailability of minerals. These days IP6 is used as food preservative [19, 20]. Recently, IP6 was found to be an alternative root canal chelating agent that has the potential to replace EDTA as the former was effective in removing the smear layer while being more biocompatible to osteoblast cells [21].

To date, no study has evaluated the antimicrobial effect of IP6 on E. faecalis. Thus the aims of this study were to test the antimicrobial efficacy of this agent on E. faecalis and compare it to that of clinically used irrigants such as NaOCl, EDTA, PA and CHX.

Materials and Methods

Bacterial strain. The microorganism used in this experiment was lyophilized standard strain Enterococcus faecalis (ATCC 29212) (Microbiologics, St. Cloud, USA). It was revived as per the instructions of the Microbiologics. The strain was maintained at -80°C in Trypticase Soya Broth (TSB).

Test materials. The test materials used in this study were PA (Scharlab S.L, Sentment, Spain), CHX (Sigma Aldrich, Saint Louis, USA), EDTA (Ultradent Products, Utah, USA), NaOCl (Iktmaliah Trading Est., Riyadh, KSA) and IP6 ( Wako pure chemicals, Osaka, Japan). All media used in this study were purchased from SPML (Saudi Prepared Media Laboratory, Riyadh, Saudi Arabia).

Disk diffusion test. The antimicrobial properties of 37% PA, 2% CHX, 18% EDTA, 5% NaOCl and 5% IP6 on Enterococcus faecalis were determined using the Disk Diffusion Method according to the Clinical and Laboratory Standards Institute guidelines (CLSI M02-11)[22]. Eighteen Mueller Hinton Agar plates (MHA) were inoculated with standardized inoculum. The standardized inoculum was prepared using the Direct Colony Suspension Method.
Isolated colonies from 24 hours Brain Heart Infusion (BHI) agar plates were picked with a sterile swab and transferred into Mueller Hinton Broth (MHB). The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard using Densicheck™ plus instrument (bioMérieux Inc., Durham, USA). This standard is equivalent to 1.5×10^8 CFU/ml. Ten mm sterile paper disks (Sigma Aldrich, Saint Louis, USA) were then saturated with the test solutions and transferred to its designated plates. Each plate received one test agent and one plate received a disk with sterile water (control). All seeded plates were incubated at 37°C for 18 hrs. The resulting zones of inhibition were measured using millimetre scale. All tests were carried out in triplicates.

**Minimum inhibitory concentrations**

Minum inhibitory concentration (MIC) of PA, CHX, EDTA, NaOCl and PI6 were done by the Broth Macrodilution Method according to the Clinical and Laboratory Standards Institute guidelines (CLSI M07-A9)[23].

**Minimal bactericidal concentration.** For the determination of MBC, 10µl from the tubes where there was no visible growth in the MIC experiment were subcultured onto BHI agar plates and incubated for 24 hours at 37°C. The MBC was read as the lowest concentration to kill 99.9% of the initial inoculums [24]. Growth control was subcultured for purity check. The negative controls were also subcultured as a sterility check.

**Preparing the test materials.** Twofold dilutions of the test materials were prepared. Nine sterile tubes for each reagent were labelled from 1 to 7; the last two were labelled with positive control and negative control. 1 ml of Mueller Hinton broth (MHB) was added in each tube then 1 ml of each stock solution (74% PA, 20% CHX, 18% EDTA, 6% NaOCl and 20% PI6) was added to its number 1 tube and negative control tube only. After well mixing tube one, 1 ml of this mixture was transferred to tube 2, after well mixing, 1 ml was transferred from tube 2 to tube 3. This was repeated up to tube no 7. From tube 7 1ml was discarded. The positive controls tubes did not receive reagents.

**Inoculum preparation.** Standardized inoculum was prepared using the Direct Colony Suspension Method. Isolated colonies from 24 hours Brain heart infusion (BHI) agar plates were picked with a sterile swab and transferred into Mueller Hinton Broth (MHB). The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard using Densicheck™. This standard is equivalent to 1.5×10^8 CFU/ml. The 0.5 McFarland suspension was then diluted 1:150, resulting in a tube containing approximately 1×10^6 CFU/ml. One ml of the above suspension was added to each tube containing 1 ml of the reagents in the dilution series, except the negative control tube. This resulted in 1:2 dilution of each reagent concentration and brought the final inoculum concentration to 5×10^5 CFU/ml. All tubes were covered loosely with caps and incubated at 37°C for 18 hours. After the incubation period; the tubes were examined for growth. The lowest dilution of each agent preventing the appearance of visible turbidity was considered as MIC which is bacteriostatic for the test organism. Purity check of the inoculum suspension was done by subculturing onto BHI agar. All Tests were duplicated.

**Statistical analysis.** Results obtained from disk diffusion test were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) and post hoc comparison were used to compare the mean of zones of inhibition of the tested groups. A P-value of less than 0.05 was considered to be statistically significant.

**Results**

The inhibition zones diameters of all materials against E.faecalis (ATCC 29212) are shown in Table 1. There was a statistical significant difference between the groups. PA showed a statistically sig-
significant larger zone when compared to other tested materials \((P< 0.05)\). There was no statistical significant difference between NaOCl, EDTA and CHX \((P=0.098)\). IP6 showed a statistically smaller zone of inhibition when compared to all groups \((P< 0.05)\). The MIC and MBC values obtained are reported in Table 2. These are expressed as both a percentage ratio according to the stock solution and in fold dilution (Table 2). CHX was excluded from MIC test as immediate precipitation and turbidity of CHX occurred after CHX and MHB were mixed. The recorded MIC/MBC values for IP6 against \(E. faecalis\) were 0.156%, 0.625%; respectively, indicating it has both bacteriostatic and bactericidal activities. NaOCl showed the lowest MIC value while PA showed the highest MIC value. All materials showed bactericidal activity except EDTA.

### Discussion

Enterococcus faecalis is a facultative anaerobic gram positive bacterium. It is the main microorganism involved in persistent endodontic infections. Elimination of this bacterium from root canal is essential in endodontic procedure. This study was aimed to evaluate the antibacterial effect of IP6 on \(E. faecalis\), by disk diffusion, MIC and MBC in comparison with clinically used irrigants.

Routine microbiological tests such as disk diffusion and broth dilution are commonly used to assess the antibacterial effect. Disk diffusion measures the inhibition zone size which varies with the molecular characteristics of different agents; therefore different agents acting on the same organisms cannot be compared by their inhibition zone sizes [25], and as reported by Moreno et al. [26] the absence of an inhibition zone did not necessarily mean the compound was inactive, especially for less polar compounds, which have low diffusion rate in culture medium. This might explain some of the results obtained in this study in which IP6 showed the smallest zone of inhibition against \(E. faecalis\) in disk diffusion test; however, its MIC and MBC values were comparable or even lower to other tested materials. Therefore, diffusion method is not always reliable for determining the antimicrobial activity of plant extract [27]. Broth macrodilution method, which is direct and close contact between the test microorganism and materials, was used in this study to confirm the bacterial inactivation [27] and to overcome the aforementioned limitations.

### Table 1. Mean and Standard Deviation (SD) of the inhibition zones diameters of all materials against \(E. faecalis\) using a millimeter (mm) scale.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Mean</th>
<th>SD</th>
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<tbody>
<tr>
<td>PA 37%</td>
<td>39.00</td>
<td>1.73</td>
</tr>
<tr>
<td>CHX 2%</td>
<td>29.66</td>
<td>0.57</td>
</tr>
<tr>
<td>EDTA 18%</td>
<td>31.33</td>
<td>4.04</td>
</tr>
<tr>
<td>NaOCl (5%)</td>
<td>26.33</td>
<td>1.52</td>
</tr>
<tr>
<td>IP6 (5%)</td>
<td>17</td>
<td>1</td>
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<td>-ve control</td>
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</tbody>
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### Table 2. MIC and MBC of all materials obtained using the macrodilution broth method (MICs and MBCs are given in percentage according to the stock concentrations and in fold dilutions).

<table>
<thead>
<tr>
<th>Materials</th>
<th>MIC</th>
<th>MBC</th>
</tr>
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<tbody>
<tr>
<td>PA (Stock: 74%)</td>
<td>0.578% (1/128)</td>
<td>4.6% (1/16)</td>
</tr>
<tr>
<td>EDTA (Stock: 18%)</td>
<td>0.14% (1/128)</td>
<td>R¹</td>
</tr>
<tr>
<td>NaOCl (Stock: 6%)</td>
<td>0.093% (1/64)</td>
<td>0.375% (1/16)</td>
</tr>
<tr>
<td>IP6 (Stock: 20%)</td>
<td>0.156% (1/128)</td>
<td>0.625% (1/32)</td>
</tr>
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</table>

¹ R: resistant to all dilutions tested in the study.
of disk diffusion. MIC method uses serial dilutions of a solution to determine the lowest concentration of material that necessary to inhibit growth of standardized inoculum under defined conditions. In addition, the bactericidal effect of each agent was measured by culturing the clear broth of MIC tests onto agent-free solid media [25].

Bulacio et al. [28] reported a MIC value of 0.1% for NaOCl on E. faecalis which is in close agreement with the value reported in our study. The bactericidal concentration of NaOCl, found in this study, was lower than those reported by Heling et al. [29]. NaOCl is the most used endodontic irrigant nowadays[30]. Bulacio et al. [28] reported that the antimicrobial activity of NaOCl is proportional to the drug concentration, and its optimal concentration is controversial. The antimicrobial activity of NaOCl depends on the concentration of undissociated hypochlorous acid (HClO) in solution. HClO exerts its bactericidal effect by an oxidative action on sulfydryl groups of bacterial enzymes. Inhibition of enzymes causes disrupting of important metabolic reactions, and this lead to the death of bacterial cells [31]. Despite the popularity of NaOCl, it has undesirable characteristic such as cytotoxic effects when injected into the periapical tissues, a foul smell tasty, and possible allergic reactions. And as reported previously, the high resistance of E. faecalis to NaOCl may result in the failure of the root canal treatment [32, 33].

EDTA showed bacteriostatic effectiveness at 0.14% concentration in this study. Its antibacterial activity lies in its ability to change the permeability of cell membrane [37]. EDTA showed no bactericidal activity against E. faecalis in this study and this finding is in agreement with previously published reports [38-40]. Lack of bactericidal activity of EDTA against E. faecalis is considered as a limitation to its use since bactericidal agents are preferable to be used in root canal treatment due to the anatomy of the root canal and the poor host defence mechanism in that area.

In this study, PA showed inhibition effect against E. faecalis in MIC test at a concentration of 0.578% and the MBC value was 4.6%. Arias-Moliz et al. [38] reported that MBC value for PA was 2.5%. This disagreement can be attributed to the difference of the experiment nature. Antibacterial activity of PA solution is derived from the release of hydrogen ion, which would inhibit bacterial metabolism [41].

IP6 is organic acid, natural extract from rice bran. Kim et al. [42] reported that the bactericidal effects of IP6 were much greater than those of other organic acids under the same experimental conditions. The antimicrobial effect of organic acids yields by the weak acid theory [43], but due to the unique structure of IP6 and a wide acidity range, the mechanism of the antimicrobial activity of PA is likely to be different [44, 45]. The bactericidal activity and the mode of action of IP6 have not been studied in details till this date. In this study, MIC tests showed that the effective IP6 concentration was lower than those of PA, 0.156% vs. 0.578%, respectively. The MIC value of IP6 was close to the MIC value of EDTA and NaOCl (0.14% and 0.093% respectively). IP6 showed bactericidal activity against E. faecalis at 0.625%.

This study suggests that IP6 is an effective agent against E. faecalis based on the obtained MIC and MBC values which were comparable or even lower than the ones obtained for the currently used irrigation solutions.
Acknowledgments

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