Evaluation of the antimicrobial efficacy of 20% Punica granatum, 0.2% chlorhexidine gluconate, and 2.5% sodium hypochlorite used alone or in combinations against Enterococcus faecalis: An in-vitro study

Laxmish Mallya, Ramya Shenoy, Kundabala Mala, Suchitra Shenoy

Departments of Conservative Dentistry and Endodontics and 1Public Health Dentistry, Manipal College of Dental Sciences, Affiliated to Manipal Academy of Higher Education, 2Department of Microbiology, Kasturba Medical College, Affiliated to Manipal Academy of Higher Education, Mangalore, Karnataka, India

Abstract

Objectives: The study was aimed at evaluating the antimicrobial efficacy of 20% Punica granatum, 0.2% chlorhexidine (CHX) gluconate, and 2.5% sodium hypochlorite used alone or in combinations against Enterococcus faecalis.

Materials and Methods: Aqueous extract of pomegranate peel was prepared in the Pharmacology Departmental Laboratory. A total of 240 wells were prepared (40 for each group) with 5 wells per with a diameter of 6 mm and depth of 4 mm at equidistant from each other. Using a pipette, each well was filled with 50 μl of the test irrigant solution. CHX (0.2%), 2.5% sodium hypochlorite, and aqueous extract of pomegranate peel and their combinations were tested as root canal irrigants against a standard strain of E. faecalis (ATCC 29212) on sheep blood agar plate by calculating the zones of inhibition. The mean diameter of zones was calculated and tabulated. Data were analyzed using one-way analysis of variance and Tukey’s post hoc test. Descriptive statistics was obtained using SPSS software (version 11.5) with P established at < 0.05.

Results: Combination of Punica granatum with sodium hypochlorite and CHX showed maximum mean zones of inhibition with mean of 23.9 and 25.7 mm, respectively, and showed significantly better results than all other groups either irrigants used alone or in combinations.

Conclusions: Punica granatum and CHX was proved to be a very good combination among experimental groups against E. faecalis, and sodium hypochlorite was least effective against E. Faecalis.

Keywords: Chlorhexidine gluconate; Enterococcus faecalis; Punica granatum; sodium hypochlorite

INTRODUCTION

The success of root canal therapy mainly depends on the complete disinfection of the infected root canal system. The endodontic therapy can fail if bacteria persist in the canal or periradicular region; if the canal is obturated inadequately; or if there are overextensions of root filling materials, improper and leaky coronal seal, missed canals, irreparable mishaps, and iatrogenic procedural errors.[1] Persistence of pathogenic bacteria and their toxins and byproducts play a critical role in infecting the root canal system including the radicular dentinal tubules, in primary as well as failed

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pulp space therapy. Several researchers have recovered numerous species of anaerobic and facultative anaerobic bacteria from failed root canal cases. Seven major phyla of microbes associated with endodontic infections are Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes, and Synergistetes. In addition, Fungi, Archaea, and viruses are found. The Gram-negative anaerobic rods found in the canal are Fusobacterium nucleatum, Prevotella spp., and Campylobacter rectus. The Gram-positive bacteria found are Streptococci, Lactobacilli, Staphylococci, Enterococcus faecalis, Olsenella uli, Parvimonas micra, Pseudoramibacter alactolyticus, Propionibacterium spp., Actinomyces spp., Bifidobacterium spp., and Eubacterium spp. Sometimes, yeasts, commonly Candida albicans, are also found in small amounts, among which E. faecalis was found to be the most prevalent bacterium that plays a major role in the persistence of periradicular lesions even after root canal therapy. Nearly 22%-77% of endodontic failure cases found that E. faecalis could survive in the root canal as a single organism or as a major component of the flora. Due to its ability at biofilm formation, E. faecalis can survive severe conditions, making them more resistant to phagocytosis, antibodies, and antimicrobial agents. Enterococci have capacity to form biofilm which make them more resistant to most of the antibacterial agents. Among the Enterococci, E. faecalis is considered to be one of the most resistant species which is found in the infected root canals, and is often associated with failed endodontic treatment. 

Disinfection of the root canal system is done by chemo-mechanical preparation which involves precise instrumentation to reach all the nooks and corners of the canal system by the chemical irrigants, not only to eradicate all the microbes, but also to remove the biofilm. Hence, irrigants should have the ability to dissolve pulp tissue debris, to remove the smear layer and biofilm, and also be antimicrobial in nature. Currently, sodium hypochlorite is the irrigant which is preferred by most clinicians for various reasons such as pulp-dissolving capacity, eradication of microbes from biofilm, and excellent antimicrobial capacity. Moreover, it is a very good irrigant for eliminating even spore-forming organisms, thus disinfecting the canal system completely. Sodium hypochlorite has proved to be an effective antimicrobial agent against E. faecalis. Unfortunately, its adverse effect on vital periradicular tissues still persists. Other irrigants used by most clinicians are bis-bisguanide, chlorhexidine (CHX) gluconate, which is a very good antimicrobial agent and proved to be effective against E. faecalis. It is supposed to have lower tissue toxicity compared to sodium hypochlorite. However, CHX lacks the tissue-dissolving property because of that it has failed to replace sodium hypochlorite. As CHX does not remove the smear layer, it cannot replace ethylenediaminetetraacetic acid too. 

Various civilizations have been using medicinal herbs (phytomedicine) for the treatment of dental caries and periodontal disease. Punica granatum which is an extract of pomegranate fruit peel (PPE) has been used effectively in Iranian medicine for its bactericidal, antifungal, antiviral, immune modulation, and anthelmintic effects. The active ingredients of PPE are flavonoids, tannins, and other phenolic compounds. PPE has a shelf-life of 2 months due to antimicrobial effect of the extract. In the prevention of some of the oral diseases such as caries and gingivitis/periodontitis, polyphenols and tannins have been tried with encouraging results which are extracted from different natural products such as Punica granatum peel extracts, Acacia nilotica, Cuminum cyminum, Foeniculum vulgare, and Coffea canephora aqueous extract. However, the impact of this extract from the Punica granatum peel on the usual endodontic pathogenic bacteria involved in root canal infections has not been reported in literature.

Hence, the present in-vitro study was conducted to evaluate the antimicrobial effect of 20% Punica granatum, against E. faecalis when used alone and also in combinations with other routine endodontic irrigating solutions.

**MATERIALS AND METHODS**

**Preparation of extract**

*Punica granatum* (pomegranate) fruits were obtained. Following thorough washing, of these fruits, the mesocarp of pomegranate was separated and dried. Preparation of aqueous extract of pomegranate peel was done in the laboratory of pharmacology based on a previous study by Pai et al. Initially, 10 g of the material was boiled in 100-ml sterile distilled water for 15 min on a low flame, allowed to cool for 45 min. To remove any impurities, contents of the flasks were filtered following cooing with double filter paper and sterile filters. To obtain 20%, each extract was diluted with sterile water, then stored in a sterile container in a refrigerator, and transported to the department of microbiology for microbiological assays. CHX (0.2%) and 2.5% sodium hypochlorite were the other two test irrigants used for comparison. All the samples were stored in the refrigerator at 4°C following which the samples were transported to the department of microbiology for analyses. The standard strain of *E. faecalis* (ATCC 29212) was used as a test strain. Using a wire loop, from an agar medium, morphologically similar colonies were picked up. The growth of the strain was transported to a test tube containing 4 ml of sterile peptone water. According to the standard protocol of inoculum preparation, sheep blood agar (Hi-Media Pvt. Ltd, Mumbai, Maharashtra, India) was used to evaluate the antimicrobial activity against *E. faecalis*. The McFarland tube lawn culture was done following adjusting the inoculums to 0.5 on blood agar plates. A total of 240 wells were prepared (40 for each group) with 5 wells per plate with a diameter of 6 mm and
depth of 4 mm at equidistant from each other. Using a pipette, each well was filled with 50 µl of the test irrigant solutions.

The samples were divided into the following groups:

- **Group A (POME)** – 20% pomegranate peel extract
- **Group B (HYPO)** – 2.5% Sodium hypochlorite
- **Group C (CHX)** – 0.2% CHX gluconate
- **Group D (POME+HYPO)** – Combination of 20% pomegranate peel extract and 2.5% sodium hypochlorite
- **Group E (POME+CHX)** – Combination of 20% pomegranate peel and 0.2% CHX gluconate
- **Group F (HYPO+CHX)** – Combination of 2.5% sodium hypochlorite and 0.2% CHX gluconate.

The agar plates were incubated in a CO₂ incubator (NuAire Inc., Plymouth, MN, USA) at 37°C for 48 h. Using a millimeter scale, the zones of inhibition were measured; the scale was passed through the diameter of the zones formed and to the point of complete inhibition of growth on either side. The mean diameter was calculated and tabulated. Data were analyzed using one-way analysis of variance (ANOVA) and Tukey’s post hoc test. Descriptive statistics were obtained using SPSS software (version 11.5) with P established at <0.05. Finally, conclusions were drawn from these analyses.

**RESULTS AND ANALYSIS**

A total of six groups of irrigating solutions were checked for efficacy on *E. faecalis*. The diameter of zones of inhibition is tabulated for all the groups with their mean and standard deviation [Table 1]. Using ANOVA and Tukey’s post hoc test, data were statistically analyzed with significance level at P < 0.05 [Table 2].

Among all the experimental groups, the best result with the largest zone of inhibition was obtained with statistically significant difference by the combination of *Punica granatum* and CHX (mean zone 25.7 mm). The zone of inhibition was least in group where only sodium hypochlorite was used (mean zone 15.8950) [Table 1]. All the groups showed statistically significant difference with other experimental groups except CHX with the combination of CHX and NaOCl [Table 3]. Combination of *Punica granatum* with sodium hypochlorite and CHX showed maximum mean zones of inhibition with mean of 23.9 and 25.7 mm, respectively, and showed statistically significantly better results than all other groups either irrigants used alone or in combinations (P < 0.05) [Table 2].

**DISCUSSION**

In the present study, the combination of *Punica granatum* and CHX showed the largest zone of irrigation against *E. faecalis*, among all the experimental irrigants. This could be because of the synergistic action of *Punica granatum* and CHX. Numerous studies have concluded that CHX is very effective against *E. faecalis*. It is a bis-biguanide which is an excellent antimicrobial agent that is effective against *E. faecalis*. *Punica granatum* has antiviral, bactericidal, and antifungal properties due to the immune modulation and anthelmintic actions of pomegranate. Polyphenols and tannin extracts from *Punica granatum* help prevent biofilm-related oral diseases such as dental caries and gingivitis/periodontitis. Hence, *Punica granatum* must have acted on endodontic biofilms too.

Adding *Punica granatum* to CHX must have enhanced the antibacterial effect of CHX against *E. faecalis*. Hence, it has resulted in larger zone of inhibition. Moreover, combination of *Punica granatum* along with sodium hypochlorite and CHX showed better results than when these irrigants were used alone as well as combination of hypochlorite and CHX. This shows that when we combine *Punica granatum* with routinely used irrigants such as sodium hypochlorite and CHX, it enhances the effect with synergistic action. This could be because of the disturbance of polyglycans synthesis due to the presence of ellagittannin and punicalagin which interfere the adherence mechanism of these organisms to the dental surface. Moreover, the tannin present in PPE penetrates the cell wall of the microorganisms and binds to its surface, leading to the precipitation of proteins and suppressing enzyme such as glycosyl transferase which is a key enzyme for the breakdown of carbohydrates, which provides nutrition to organisms. The phenolic compounds present in PPE make the microorganisms difficult to survive by bacterial cell wall disruption. Antioxidant property of Anthocyanins present in the pomegranate is highly bioactive. When PPE...
Table 3: Inter‑group comparison of mean difference in the zone of inhibition

| Group           | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval |
|-----------------|-----------------------|------------|------|-------------------------|
| **POME**        |                       |            |      |                         |
| HYPO            | 2.98250*              | 0.01749    |      | 2.9322 - 3.0328         |
| CHX             | 0.000                 | 0.000      |      | -0.6322 - 0.6322       |
| POME + HYPO     | -5.02250*             | 0.01749    |      | -5.0728 - 4.9722       |
| POME + CHX      | -6.82250*             | 0.01749    |      | -6.8728 - 6.7722       |
| HYPO + CHX      | -0.72350              | 0.01749    |      | -0.7738 - 0.6732       |
| **HYPO**        |                       |            |      |                         |
| CHX             | -3.66500*             | 0.01749    |      | -3.7153 - 3.6147       |
| POME + HYPO     | -8.00500*             | 0.01749    |      | -8.0553 - 7.9547       |
| POME + CHX      | -9.80500*             | 0.01749    |      | -9.8553 - 9.7547       |
| HYPO + CHX      | -3.70600*             | 0.01749    |      | -3.7563 - 3.6557       |
| **CHX**         |                       |            |      |                         |
| POME + HYPO     | -4.34000*             | 0.01749    |      | -4.3903 - 4.2897       |
| POME + CHX      | -6.14000*             | 0.01749    |      | -6.1903 - 6.0897       |
| HYPO + CHX      | -0.04100              | 0.01749    |      | -0.0913 - 0.0093       |
| POME + HYPO     | -1.80000*             | 0.01749    |      | -1.8503 - 1.7497       |
| POME + CHX      | 4.29900*              | 0.01749    |      | 4.2487 - 4.3493        |
| **POME + CHX**  | 6.09900*              | 0.01749    |      | 6.0487 - 6.1493        |

is combined with hypochlorite and CHX, the antibacterial property must have enhanced.[21] Further in‑vivo studies are required to confirm the results in a polymicrobial endodontic environment of root canal system.

CONCLUSIONS

Within the limitations of this study, it can be concluded that,
1. *Punica granatum* and CHX was the best combination of irrigants among experimental groups against *E. faecalis* followed by the combination of *Punica granatum* and sodium hypochlorite
2. Sodium hypochlorite was least effective against *E. faecalis* among the experimental groups.

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Conflicts of interest

There are no conflicts of interest.

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