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# Comparison of antimicrobial activity of two chelating agents chitosan and etidronate against *Enterococcus faecalis* using agar diffusion test

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**Abstract**
Etidronate and chitosan are relatively newer chelating agents used in endodontic practice. Little is known about their additional antimicrobial properties. In this study, we compared the activity of etidronate and chitosan against *Enterococcus faecalis* using agar diffusion test. Zones of inhibition were compared between the two agents, positive control (2% chlorhexidine) and negative control (1% acetic acid). The antibacterial activity of 18% etidronate against *E. faecalis* was found to be significantly superior to that of 0.2% chitosan.

**Keywords:** Etidronate, Chitosan, Antibacterial

## 1. Introduction
The goal of cleaning the root canal system is to eliminate harmful agents such as micro-organisms and necrotic pulp tissue remnants. The smear layer formed after endodontic instrumentation should be completely removed from the surface of the root canal wall as it can provide an avenue for leakage. It may also harbour micro-organisms by forming biofilms. Chelating agents primarily act on the inorganic component of smear layer, aiding in its removal. Etidronate and chitosan are two chelating agents introduced recently in endodontics.
Silva *et al* have shown that chitosan at 0.2% concentration removes smear layer effectively [1]. Chitosan is also believed to have additional antibacterial and antifungal effects [2]. Ballal *et al* compared the antibacterial activities of chlorhexidine, 2% chitosan gel and their combination and found the latter to have maximal action [3]. However, the antimicrobial efficacy of chitosan at the very low concentration of 0.2% used for chelation has not been evaluated.
Recently, hydroxy ethylidene bisphosphonate (HEBP), also known as etidronate, has been studied as a milder chelating agent. It has traditionally been used as a systemic drug for the treatment of Paget's disease and osteoporosis. De-Deus *et al* found that 18% etidronate showed chelating ability similar to 17% EDTA after a 3 minutes application time [4]. In addition, some studies have revealed that bisphosphonates have antimalarial activity and also inhibit *Escherichia coli (E coli)* [5, 6].
This study was done to compare the antibacterial activity of the above two chelating agents 18% etidronate and 0.2% chitosan against *Enterococcus faecalis (E. faecalis)* using agar diffusion test. *E. faecalis* has been consistently identified as the species most commonly recovered from root canals of teeth following failed endodontic treatment [7, 8]. Commonly used irrigants such as sodium hypochlorite and EDTA have inadequate action against this bacterium. *E. faecalis* is thus notorious for surviving in the root canal system and invading dentinal tubules. For these reasons, *E. faecalis* was chosen to test the antibacterial activity of the chelating agents in the current study.

## 2. Methods
0.2% chitosan was prepared by mixing 200 mg of chitosan (India Sea Foods Inc., Cochin, India) in 100 ml of 1% acetic acid. This solution was agitated for 2 hours in a magnetic stirrer. 18% HEBP was prepared by mixing 18 grams of etidronate powder (Sigma Aldrich Chemical Distributors, Bangalore, India) in 100 ml of distilled water.
Agar diffusion test was performed according to previously described standard methodology [3]. Briefly, *E. faecalis* ATCC 29212 strain was sub-cultured in 2 ml of brain heart infusion (BHI)

broth and incubated at 37 degrees centigrade in a candle extinction jar for 18 hours. The culture was adjusted to 0.5 McFarland's opacity tubes. 25 microlitres of the adjusted culture was added to 30 ml of freshly prepared BHI agar, mixed well, poured into a sterile petridish and allowed to set. Using sterile templates 5 wells of 6 mm diameter were cut in the medium and 100 microlitres of the following reagents were added: 0.2% chitosan, 18% etidronate, 2% chlorhexidine (positive control) and 1% acetic acid (negative control). In a short pilot study, the zone of inhibition of 1% acetic acid, which is the diluent for preparation of 0.2% chitosan solution, was compared with that of distilled water. Both 1% acetic acid and distilled water were found to have minimal antibacterial activity, with similar zones of inhibition. Hence 1% acetic acid was used as the negative control for this study.

Plate was incubated at 37 degree centigrade in a candle extinction jar for 18 hours. The zone of inhibition around the wells was measured in mm using a Vernier calliper. The test was done in 9 replicates.

Statistical analysis was performed with Minitab version 17 statistical software (Minitab Ltd, Coventry, UK). Groups were evaluated using one-way ANOVA and Kruskal-Wallis test. Pairwise comparison was done using Newman Keuls multiple post hoc procedure and Mann-Whitney U test. All p values were two-sided and $p<0.05$ was considered statistically significant.

## 3. Results

2% chlorhexidine and 18% etidronate demonstrated large zones of inhibition; the representative zones for each of the four groups are as shown (Figure 1). 18% etidronate had zones of inhibition much larger than those of 0.2% chitosan (table I). The zones of inhibition values were found to be slightly higher for 18% etidronate when compared with 2% chlorhexidine, the positive control. 0.2% chitosan showed zones of inhibition barely larger than 1% acetic acid, the negative control (table I). Mean, standard deviation, standard error and coefficient of variation for each group are as shown (table II). Mean values were 19.61, 7.94, 21.83 and 8.67 mm respectively for 2% chlorhexidine, 1% acetic acid, 18% etidronate and 0.2% chitosan. Pair-wise comparative analysis showed statistically significant differences between each of the four groups (table II).

As would be expected, significant difference was evident between the zones of inhibition of the positive and negative controls, with 2% chlorhexidine showing considerably higher mean value (19.61 mm) against 1% acetic acid (7.94 mm) ($p=0.0003$). By Mann-Whitney U-test, the zones of inhibition were significantly higher for 18% etidronate when compared with 2% chlorhexidine ($p=0.0003$), 1% acetic acid ($p=0.0003$) and 0.2% chitosan ($p=0.0003$) (table III). 0.2% chitosan was significantly better than 1% glacial acetic acid ($p=0.0062$), but was inferior when compared with 2% chlorhexidine and 18% etidronate (table III).

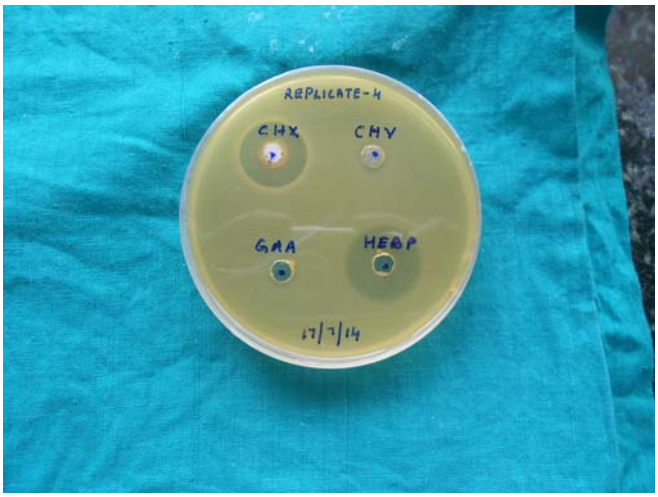


**Fig 1:** Representative image for zones of inhibition for the four groups. CHX, 2% chlorhexidine; CHY, 0.2% chitosan; GAA, 1% acetic acid; HEBP, 18% etidronate

**Table I:** Zones of inhibition (in mm)

| Replicates | 2% Chlorhexidine | 1% Acetic acid | 18% Etidronate | 0.2% Chitosan |
|---|---|---|---|---|
| 1 | 19.5 | 7 | 21.5 | 8.5 |
| 2 | 20 | 8.5 | 22.5 | 9 |
| 3 | 19.5 | 8 | 22 | 9 |
| 4 | 19 | 7.5 | 22 | 8 |
| 5 | 20 | 8 | 21 | 8.5 |
| 6 | 20.5 | 8.5 | 22.5 | 8.5 |
| 7 | 19.5 | 8 | 21.5 | 9 |
| 8 | 19.5 | 8 | 22.5 | 8.5 |
| 9 | 19 | 8 | 21 | 8.5 |

**Table II:** Pair wise comparison of four groups with zones of inhibition (in mm) by Newman Keuls multiple post hoc procedures

| Groups | 2% Chlorhexidine | 1% Acetic acid | 18% Etidronate | 0.2% Chitosan |
|---|---|---|---|---|
| Mean | 19.61 | 7.94 | 21.83 | 8.61 |
| Standard deviation | 0.49 | 0.46 | 0.61 | 0.33 |
| Standard error | 0.16 | 0.15 | 0.2 | 0.11 |
| Coefficient of variation | 2.48 | 5.84 | 2.8 | 3.87 |
| 2% Chlorhexidine | - | | | |
| 1% Acetic acid | p=0.0001* | - | | |
| 18% Etidronate | p=0.0001* | p=0.0002* | - | |
| 0.2% Chitosan | p=0.0001* | p=0.0065* | p=0.0001* | - |

*p<0.05

**Table III:** Pair wise comparison of four groups with zones of inhibition (in mm) by Mann-Whitney U test

| Groups | Mean | SD | Median | Sum of ranks | U-value | Z-value | p-value |
|---|---|---|---|---|---|---|---|
| 2% Chlorhexidine | 19.61 | 0.49 | 19.50 | 126.00 | | | |
| 1% Acetic acid | 7.94 | 0.46 | 8.00 | 45.00 | 0.00 | -3.5762 | 0.0003* |
| 2% Chlorhexidine | 19.61 | 0.49 | 19.50 | 45.00 | | | |
| 18% Etidronate | 21.83 | 0.61 | 22.00 | 126.00 | 0.00 | -3.5762 | 0.0003* |
| 2% Chlorhexidine | 19.61 | 0.49 | 19.50 | 126.00 | | | |
| 0.2% Chitosan | 8.61 | 0.33 | 8.50 | 45.00 | 0.00 | -3.5762 | 0.0003* |
| 1% Acetic acid | 7.94 | 0.46 | 8.00 | 45.00 | | | |
| 18% Etidronate | 21.83 | 0.61 | 22.00 | 126.00 | 0.00 | -3.5762 | 0.0003* |
| 1% Acetic acid | 7.94 | 0.46 | 8.00 | 54.50 | | | |
| 0.2% Chitosan | 8.61 | 0.33 | 8.50 | 116.50 | 9.50 | -2.7374 | 0.0062* |
| 18% Etidronate | 21.83 | 0.61 | 22.00 | 126.00 | | | |
| 0.2% Chitosan | 8.61 | 0.33 | 8.50 | 45.00 | 0.00 | -3.5762 | 0.0003* |

*p<0.05

## 4. Discussion

An ideal irrigant should possess tissue dissolving property, ability to remove smear layer and antibacterial activity. At present no single irrigant combines all these ideal characteristics even when used at a lower pH, increased temperature or with surfactants to increase their wetting efficacy. Chitosan and etidronate at concentrations of 0.2% and 18% respectively have been shown to result in good smear layer removal [9, 10]. Neither of these agents possess adequate tissue dissolving action independently but mixing with 5.25% sodium hypochlorite has been shown to impart this property [11]. There are no studies to date comparing the bacterial inhibitory action of chitosan and etidronate head-to-head, although both chemicals are believed to have some effect.

Chitosan has broad spectrum of activity against many microbes. It has been observed to act more quickly on fungi than on bacteria. Three antibacterial mechanisms have been proposed: (i) the ionic surface interaction resulting in cell wall leakage; (ii) inhibition of mRNA and protein synthesis via penetration of chitosan into the nuclei of microorganisms; and (iii) formation of an external barrier, chelating metals and provoking the suppression of essential nutrients to microbial growth. It is likely that all these events occur simultaneously but at different intensities [12].

Bisphosphonates were initially shown to have potent activity against many protozoans [5, 13]. Later studies demonstrated additional antibacterial action, notably against *E. coli*. [14] Lujan *et al* then identified that two bisphosphonates, etidronate and clodronate, had the ability to inhibit relaxase enzyme activity and conjugative DNA transfer, thereby killing bacteria which possess antibiotic resistance property [15].

This study shows that the antibacterial activity of 18% etidronate against *E. faecalis* is significantly superior to that of 0.2% chitosan. The additional antibacterial property is a potential advantage when the former is used as a final irrigant in the root canal. A previous study has already shown that at 2% concentration, chitosan has sufficient inhibitory action against bacteria and fungi [3]. Our study demonstrates that at the lower concentration of 0.2%, which is adequate for chelating efficacy, chitosan does not have the same level of bacterial growth inhibition. Based on our results, 18% etidronate may be preferable over 0.2% chitosan in the preparation of root canals.

Chitosan is produced synthetically by the de-acetylation of chitin, which is the structural element in the exoskeleton of crustaceans. The molecular weight (MW) of chitosan and the degree of de-acetylation (DA) can potentially affect its antibacterial activity [12, 16]. In general the lower the MW and the DA, the higher will be the effectiveness on reducing microorganism growth and multiplication [12]. On average, the MW of commercially produced chitosan is between 3800-20000 Daltons. The material used for the preparation of 0.2% chitosan in this study was a 90.84% de-acetylated commercially available powder. Its MW was not known and was not tested as this was beyond the scope of the current study. It is hence likely that chitosan preparation with a different MW and/or DA may show a better antibacterial activity at 0.2% concentration and needs to be evaluated in future studies.

In conclusion, the antibacterial efficacy and chelating ability of 18% etidronate and the tissue dissolving property of 5.25% sodium hypochlorite can be combined to yield a potentially 'complete' irrigating solution, thereby precluding the need for concurrent usage of other agents such as chlorhexidine.

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