ORIGINAL RESEARCH ARTICLE

Evaluation of Antimicrobial Efficacy of Herbal Extracts (*Tridax procumbens* and *Aegle Marmelos*) and 5% Sodium Hypochlorite as Irrigants against *Enterococcus faecalis*: An *In Vitro* Study

L. R. Surender, Dinapadu Sainath, C. Rakesh Reddy, T. V. Laxmi Gayathri, B. Naveen Kumar, S. Chandrasekhar

Department of Conservative and Endodontics, SVS Institute of Dental Sciences, Appanapally, Mahabubnagar, Telangana State, India

Email for correspondence: gayathrirao9@gmail.com

ABSTRACT

Background: Oral microorganisms, which are usually opportunistic pathogens invade a root canal containing necrotic tissue and establish an infectious process causing primary endodontic infections. When root canal remains infected for long periods, the number of facultative anaerobic bacteria increases. The most common facultative anaerobic Gram-positive coccus cultured from non-healing endodontic cases is *Enterococcus* species. **Aims and Objectives:** The present study was done to evaluate the antimicrobial efficacy of *Tridax procumbens*, *Aegle marmelos*, and 5% sodium hypochlorite against *Enterococcus faecalis* biofilm formed on the tooth substrate. **Materials and Methods:** Extracted human teeth were biomechanically prepared, vertically sectioned, placed in the tissue culture wells exposing the root canal surface to *E. faecalis* to form a biofilm. At the end of the 3rd and 6th weeks, all groups were treated for 10 min with the test solutions and control and were analyzed qualitatively and quantitatively. **Results:** Qualitative assay with 3-week biofilm showed complete inhibition of bacterial growth with *T. procumbens*, *A. marmelos* and NaOCl, except saline, which showed the presence of bacterial growth. In quantitative analysis, Aegle and saline-treated tooth samples showed 1.344 ±123 CFU/mL and $186.4 \times 10^9 \pm 4.1 \times 10^9$ CFU/mL (mean and standard deviation), respectively. Qualitative assay with 6-week biofilm showed growth when treated with Tridax and marmelos whereas NaOCl has shown complete inhibition. All treated groups have shown a significant reduction of the bacterial population compared with the control group, and NaOCl showed 100% reduction. **Conclusion:** Nearly 5% sodium hypochlorite showed maximum antibacterial activity against *E. faecalis* biofilm formed on tooth substrate. *T. procumbens*, *A. marmelos* also showed statistically significant antibacterial activity.

Key words: *Aegle marmelos*, biofilm, *Enterococcus faecalis*, herbal, NaOCl, root canal irrigant, *Tridax procumbens*

Quick Response Code	Article Info:
	doi: 10.5866/2018.10.10149
	Received: 24-09-2018 **Revised:** 28-10-2018 **Accepted:** 20-11-2018 **Available Online:** 05-01-2019 (www.nacd.in)

INTRODUCTION

Oral microorganisms, which are usually opportunistic pathogens invade a root canal containing necrotic tissue and establish an infectious process causing primary endodontic infections.[1] When root canal remains infected for long periods, the number of facultative anaerobic bacteria increases.[2] The most common facultative anaerobic

Gram-positive coccus cultured from non-healing endodontic cases is *Enterococcus* species.[3-6] It is isolated in pure culture or as a major component of the flora of previously root-filled teeth with chronic apical periodontitis.[5]

This microorganism can survive even in an environment with scantly available nutrients and in which commensality with other bacteria is minimal. It grows through the formation of a biofilm, an adaptive process that enables the microorganism to tolerate in severely harsh conditions like obturated root canals.[3] *Enterococcus faecalis* can colonize the dentinal tubules and reinfect the obturated root canal as it can survive chemomechanical instrumentation and intracanal medication.[7,8]

For many years, intracanal irrigants were used as an adjunct to enhance the antimicrobial effect of cleaning and shaping in endodontics. Instrumentation, the use of irrigating solutions with antimicrobial activity remove and kills the majority of the microbial cells in the root canal; it has been shown that a small part of the flora survives.[9] Researchers have cast a sharper eye on natural products to get medicinally important compounds from plants.[10] Parts of plant such as root, stem, flower, fruit, and twigs exudates of medicinal plants represent a rich source of antimicrobial agents (Mishra *et al.* 2011). Studies reported that NaOcl was significantly efficient in reducing *E. faecalis* biofilms.[11] The unpleasant taste, high toxicity, and its inability to remove the smear layer were the main disadvantages of NaOCl.[12-14] Furthermore, an increase in antibiotic-resistant strains and side effects caused by synthetic drugs has provoked researchers toward herbal alternatives.

Plants are known to contain innumerable biologically active compounds which possess antibacterial properties. Today, nearly 88% of the global population turn to plant-derived medicines as their first line of defense for maintenance of health and combating diseases.[15] *Tridax procumbens* (T) and *Aegle marmelos* have been reported to exert antimicrobial properties suggesting their potential to be used as endodontic irrigants. However, there is a lack of sufficient supporting credentials regarding the antibacterial activity of these extracts in endodontics.[16,17]

Hence, the present *in vitro* study was aimed to evaluate the antimicrobial efficacy of *T. procumbens*, *A. marmelos*, and 5% sodium hypochlorite against *E. faecalis* biofilm formed on tooth substrate of extracted human teeth.

MATERIALS AND METHODS

A pure culture of *E. faecalis* (American Type Culture Collection [ATCC] 29212) was incubated at 37C overnight at SVS Institute of Dental Sciences, and adjusted to an optical density (OD600) of 1 with sterile broth. *T. procumbens* and *A. marmelos* extracts were made into a solution by dissolving them in 10% dimethyl sulfoxide (DMSO) (S.D. Fine Chem Pvt. Ltd, Chennai, India). The antibacterial activity of *T. procumbens*, *A. marmelos*, and 5% sodium hypochlorite (Prime Dental Products, Mumbai, India) was initially tested on planktonic cells before evaluating them against *E. faecalis* biofilm formed on tooth substrate. The antibacterial sensitivity test was performed by the disk diffusion method. Sterile blank discs (6-mm diameter; HiMedia, Mumbai, India) were impregnated with 10 mL of test solutions (Tridax, *A. marmelos*, and sodium hypochlorite).

The broth culture of *E. faecalis* was swabbed on sterile blood agar plates using sterile swabs. With the help of sterile forceps, the test solutions–incorporated discs were placed on the medium, and the plates were incubated at 37°C overnight. A disc with 10% DMSO was also included to see if it showed any significant zone of inhibition.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the test solutions were determined by the tube dilution method. Double-dilution was made from a higher dilution 100 mg/mL to a lower dilution in a series of test tubes. Each tube was inoculated with bacterial suspensions and incubated at 37°C overnight. The MIC was regarded as the lowest concentration in the series of dilutions, which did not permit the growth of the susceptible bacteria.

The subcultures were made from the tubes, which did not yield any visible turbidity (growth) in the MIC assay on freshly prepared blood agar plates. After 24 h of incubation at 37°C, the MBC was regarded as the lowest concentration of the test solution that allowed <0.1% of the original inoculum to grow on the surface of the medium. In each experiment, test solutions were tested in triplicate.

The time-kill study was done, which determined the time required for killing *E. faecalis* (ATCC 29212) by exposing the bacteria with the bactericidal

concentration of test solutions for 30 min. At regular intervals (2 min), a loop full of the sample was inoculated on an agar plate, incubated at 37°C for 24 h, and observed for growth.

Biofilm formation on tooth substrate: Single-rooted human mandibular premolars with fully formed apices were used in this study. The teeth were cleaned of superficial debris, calculus, and tissue tags and stored in normal saline to prevent dehydration before use. Each tooth was radiographed to confirm the presence of a single patent canal. The tooth specimens were sectioned below the cementoenamel junction with a diamond disc to obtain a standardized tooth length of 8 mm for the uniform specimen. The root canals were then instrumented using the crown-down technique and rotary instruments (ProTaper, Dentsply Maillefer, Ballaigues, Switzerland), and the canals were enlarged to an apical size F3. 2 mm of 3% NaOCl was used between each instrument during the cleaning and shaping procedure. All the teeth were then vertically sectioned along the midsagittal plane into two halves. The concave tooth surface was minimally grounded to achieve a flat surface to enable placement in the tissue culture wells, exposing the root canal surface to *E. faecalis* to form a biofilm.

The sectioned samples were then divided into four experimental groups. Each group consisted of 24 samples each and assigned as Group 1 (*T. procumbens*), Group 2 (*A. marmelos*), Group 3 (5% sodium hypochlorite), and Group 4 (saline). The samples were placed in the wells of tissue culture plates. The bacterium was cultured as described previously, and the wells containing tooth samples were inoculated with 2 mL of bacterial solution and incubated at 37°C.

The culture medium (BHI broth) was replaced every alternate day to avoid nutrient depletion and accumulation of toxic end products. The samples were taken from each well with a sterile paper point, inoculated onto blood agar plates, and incubated at 37 C for 24 h to check for cell viability and purity of culture. At the end of the 3rd week, all groups were treated for 10 min as follows: Group 1, immersed in 3 mL of Tridax (60 mg/mL in 10%DMSO); Group 2, immersed in 3 mL of *A. marmelos* (60 mg/mL in 10% DMSO); Group 3, immersed in 3mL of 5% NaOCl; and Group 4, immersed in 3 mL sterile saline. Then, the biofilm on the root canal portion was scraped and inoculated on blood agar plates and incubated for 24 h at 37 C for qualitative analysis where $n = 5$ for each group. The quantitative analysis was performed by vortexing the tooth samples with sterile saline for a few min followed by serial dilution method for all the groups where $n = 10$ for each group. The same procedure was repeated for all groups once again at the end of the 6th week to analyze qualitatively and quantitatively.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance and compared by the Student's t test using SPSS software (21 Version). $P < 0.05$ was considered as statistically significant.

RESULTS

Table 1 shows the zone of inhibition, MIC, and MBC of test solutions for *E. faecalis* (ATCC 29212). All test solutions have shown a significant zone of inhibition in the disk diffusion assay. Maximum inhibition was observed by 5% NaOCl followed by *T. procumbens* and Aegle. A statistically significant difference was observed between NaOCl, when compared with Tridax and *A. marmelos* ($P < 0.05$). However, there was no statistically significant difference between *T. procumbens* and *A. marmelos*. NaOCl attained 100% killing of *E. faecalis* at 2 min, whereas *T. procumbens* and *A. marmelos* took 6 min.

The qualitative assay with the 3-week biofilm on the canal portion showed complete inhibition of bacterial growth when treated with Tridax, Aegle, and NaOCl, but the samples treated with saline showed the presence of bacterial growth. In quantitative analysis, aegle and saline-treated tooth samples showed 1.344 ± 123 CFU/mL and $186.4 \times 10^9 \pm 4.1 \times 10^9$ CFU/mL (mean and standard deviation), respectively.

Table 1: Susceptibility of *E. faecalis* ATCC 29212 against the test solutions zone of inhibition, minimal inhibitory concentration, minimal bactericidal concentration of test solutions against *E. faecalis* ATCC 29212

Test solution	Zone of inhibition(mm)	MIC (%)	MBC (%)
TRIDAX	16#	2.5	5
AEGLE	14*	5	10
5% NaOCl	31#*	0.25	0.5

#$P<0.005$ with respect NaOCl and tridax, *$P<0.005$ with respect NaOCl and Aegle, ATCC: American type culture collection, *E. faecalis: Enterococcus faecalis*

Qualitative assay with the 6-week biofilm on the canal portion showed growth when treated with Tridax, and *A. marmelos* whereas NaOCl has shown complete inhibition.

Table 2 shows the bacterial population in the quantitative assay with the 6-week biofilm for Tridax, NaOCl, and saline-treated tooth samples. All treated groups have shown a significant reduction of the bacterial population compared with the control group, which showed 127.9×10^9 CFU/mL and NaOCl showed 100% reduction.

DISCUSSION

E. faecalis is the most common *Enterococcus* sp. persisting in treated root canals and are resistant to traditional antibiotics.[4,18] When *E. faecalis* grows as a biofilm, the altered genetic and metabolic processes of bacteria along with its complex matrix prevent the entry and action of several antimicrobial agents.[19] The antibiotic resistance has been found to increase up to 1.500 times when compared with planktonic cells.[20,21] Therefore, testing the effect of an antibacterial irrigant on planktonic cells will not fulfill its effectiveness in *in vivo* conditions.

Bacteria-induced dissolution of the dentin surface and the ability of *E. faecalis* to form a calcified biofilm on root canal dentin may be a factor that contributes to their persistence after endodontic treatment.[22]

It is established that the biofilm-forming capacity and its structural organization are influenced by the chemical nature of the substrate. Biofilm experiments conducted on polycarbonate or glass substrate will not provide a true indication of the bacteria-substrate interaction.[23] Hence, *E. faecalis* biofilm was formed on a tooth substrate in this study in accordance with the methodology done by Kishen *et al.*[22] All the groups were tested in direct contact with the biofilm formed on the tooth substrate at different durations (3 weeks and 6 weeks). A recent study reported that NaOCl was capable of eradicating *E. faecalis* biofilm after 1 min at a concentration of 0.00625%[24] that was grown in the Calgary biofilm forming device. However, the same concentration may not be effective on biofilm formed on tooth substrate. The antibacterial activity was directly proportional to the concentration of the test solutions. The concentration of the herbal solutions was increased because of the fact that sessile bacteria on surfaces or present within the biofilm are much less readily inactivated than planktonic cells. A biocide gradient is produced throughout the biofilm so that in thick biofilm there will be an "in-use" concentration as the biocide penetrates into the community.[23] The concentration of 60 mg/mL used in the present study was found to be effective as an antibacterial against *E. faecalis*, and further reduction in concentration, when used *in vivo*, is still feasible because the bacterial count is expected to be much less than what we have used.

Table 2: Quantitative analysis of 6-week *E. faecalis* biofilm formed on tooth substrate for different groups

Group	Number of bacteria in CFU/mL (Mean±SD)
Tridax	1123±154.4*
Aegle	1234±193*
NaOCl	0*
Control	$127.9\times10^9\pm15.4\times10^9$

*$P<0.05$ with respect to control (one-way ANOVA)

DMSO was used as a solvent for Tridax although it is readily soluble in water. DMSO is a clean, safe, highly polar, and aprotic solvent that helps in bringing out the pure properties of all the components of the herb being dissolved.[25,26] Antibacterial inertness of 10% DMSO was confirmed with the disc diffusion test.

Herbal alternatives showed promising antibacterial efficacy on 3- and 6-week biofilm with 5% sodium hypochlorite, Tridax and Aegle showed 8 log reduction compared with the control, which is considered to be highly sensitive against a particular organism.[27] Tridax and Aegle showed complete inhibition against 3 weeks biofilm, whereas they showed reduced efficacy against 6-week biofilm. This may be caused by *E. faecalis*-mediated biomineralized biofilm formation and insufficient "in-use" concentration.[22,23] Although Tridax and Aegle exhibited similar antibacterial sensitivity on *E. faecalis* planktonic cells, Tridax showed more potency on *E. faecalis* biofilm.

5% sodium hypochlorite is proven to be the best among all the groups, which exhibited excellent antibacterial activity both in 3-week and 6-week biofilm, whereas Tridax and *A. marmelos* showed complete eradication only in 3-week biofilm. NaOCl is a very caustic, nonspecific agent whose action is not limited to necrotic tissue[28] and it has deleterious effects on dentine that include reduction of the elastic modulus and the flexural strength[29,30] healing potential in plants is an ancient idea, but

in recent times it has gained renewed interest and importance. Tridax and Aegle are proven to be safe, containing active constituents that have beneficial physiologic effect apart from its curative property such as antioxidant, anti-inflammatory, and radical scavenging activity and may have an added advantage over the traditional root canal irrigants.[31-33]

T. procumbens L is a highly valuable drug and is one of the essential ingredients in most of the compound preparations included in Ayurvedic literature. The phytochemical screening revealed the presence of alkaloids, carotenoids, flavonoids (catechins and flavones), fumaric acid, fl-sitosterol, saponins, and tannins. It has antimicrobial activity against both Gram-positive and Gram-negative bacteria *T. procumbens.* The major advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far.[34]

CONCLUSION

Within the limitations of this study, 5% sodium hypochlorite showed maximum antibacterial activity against 3- and 6-week *E. faecalis* biofilm formed on tooth substrate. Tridax and Aegle showed complete eradication in 3-week biofilm. Tridax and Aegle showed statistically significant antibacterial activity against 6-week biofilm.

The use of herbal alternatives as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of NaOCl. Further research is needed to conclusively recommend herbal solutions as a root canal irrigant.

REFERENCES

1. Siqueira JF Jr., Rôças IN, Lopes HP. Patterns of microbial colonization in primary root canal infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;93:174-8.
2. Peters LB, Wesselink PR, Buijs JF, van Winkelhoff AJ. Viable bacteria in root dentinal tubules of teeth with apical periodontitis. J Endod 2001;27:76-81.
3. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85:86-93.
4. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ, *et al.* Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J 2003;36:1-11.
5. Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M. Isolation of *Enterococcus faecalis* in previously root-filled canals in a Lithuanian population. J Endod 2000;26:593-5.
6. Peciuliene V, Reynaud AH, Balciuniene I, Haapasalo M. Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. Int Endod J 2001;34:429-34.
7. Orstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Endod Dent Traumatol 1990;6:142-9.
8. Love RM. *Enterococcus faecalis*—a mechanism for its role in endodontic failure. Int Endod J 2001;34:399-405.
9. Gomes BP, Lilley JD, Drucker DB. Variations in the susceptibilities of components of the endodontic microflora to biomechanical procedures. Int Endod J 1996;29:235-41.
10. Dubey NK, Kumar R, Tripathi P. Global promotion of herbal medicine India's opportunity. Curr Sci 2004;86:37-41.
11. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative evaluation of endodontic irrigants against *E. faecalis* biofilm. J Endod 2006;32:527-31.
12. Spangberg L, Engstrom B, Langeland K. Biologic effects of dental materials. Toxicity and antimicrobial effect of endodontic antiseptics *in vitro*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1973;36:856-71.
13. McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. J Endod 1975;1:238-42.
14. Mathew ST. Risks and management of sodium hypochlorite in endodontics. Oral Hyg Health 2015;2015:3.
15. Samy RP, Ignacimuthu S, Raja DP. Preliminary screening of ethnomedicinal plants from India. J Ethnopharmacol 1999;66:235-40.
16. Kumar OA, Naidu ML. Antibacterial potential of *Tridax procumbens* L. Int J Pharm Sci 2010;1:164-73.
17. Lambole VB, Murti K, Kumar U, Sandipkumar PB, Gajera V. Phytopharmacological properties of *Aegle marmelos* as a potential medicinal tree: An overview. Int J Pharm Sci Rev Res 2010;5:67-72.
18. Pinheiro ET, Gomes BP, Ferraz CC, Teixeira FB, Zaia AA, Souza Filho FJ, *et al.* Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. Oral Microbiol Immunol 2003;18:100-3.
19. Gordon CA, Hodges NA, Marriott C. Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. J Antimicrob Chemother 1988;22:667-74.
20. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001;9:34-9.
21. Socransky SS, Haffajee AD. Dental biofilms: Difficult therapeutic targets. Periodontol 2000 2002;28:12-55.
22. Kishen A, George S, Kumar R. *Enterococcus faecalis*-mediated biomineralized biofilm formation on root canal dentine *in vitro*. J Biomed Mater Res A 2006;77:406-15.
23. McBain AJ, Gilbert P, Allison DG. Biofilms and biocides: Are there implications for antibiotic resistance? Rev Environ Sci Technol 2003;2:141-6.
24. Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, Baca P. *Enterococcus faecalis* biofilms eradication by root canal irrigants. J Endod 2009;35:711-4.

25. Jacob SW, Herschler R. Biological actions of dimethyl sulfoxide. Ann N Y Acad Sci 1975;243:1-508.

26. de la Torre JC. Biological actions and medical applications of dimethyl sulfoxide. Ann N Y Acad Sci 1983;411:1-404.

27. NCCLS. Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. Wayne, PA: NCCLS; 2005.

28. Seltzer S, Farber PA. Microbiologic factors in endodontology. Oral Surg Oral Med Oral Pathol 1994;78:634-45.

29. Sim TP, Knowles JC, Ng YL, Shelton J, Gulabivala K. Effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. Int Endod J 2001;34:120-32.

30. Grigoratos D, Knowles J, Ng YL, Gulabivala K. Effect of exposing dentine to sodium hypochlorite and calcium hydroxide on its flexural strength and elastic modulus. Int Endod J 2001;34:113-9.

31. Rao KV, Sekar DK, Kumar G, Karthik L. A review on pharmacological and phytochemical properties of *Aegle marmelos*(L.)Corr.Serr.(Rutaceae).AsianJPlantSciRes2011; 1:8-17.

32. Rasool M, Sabina EP. Antiinflammatory effect of the Indian ayurvedic herbal formulation triphala on adjuvant-induced arthritis in mice. Phytother Res 2007;21:889-94.

33. Abascal K, Yarnell E. Herbs and drug resistance. Part 2-clinical implications of research on microbial resistance to antibiotics. Altern Complement Ther 2002; 8:284-90.

34. Prabhakar J, Senthilkumar M, Priya MS, Mahalakshmi K, Sebgal PK, Sukumaran VG. Evaluation of antimicrobial efficacy of herbal alternatives (Triphala and Green tea polyphenols), MTAD and 5% sodium hypochloride against *Enterococcus faecalis* biofilm formed on tooth substrate: An *in vitro* study. J Endod 2010;36:83-6.