Root Canal Renaissance- A Comparative Evaluation of Herbal Extracts as Alternative Irrigants in Eradicating Enterococcus Faecalis: An in Vitro Study

Dr. Tanu Tewari[1*], Dr. Pranjal Jain[2], Dr. Vishesh Gupta[3], Dr. Megna Chauhan[4], Dr. Gunja Verma[5], Pro.(Dr.) Praveen Singh Samant[6]

[1*]Associate Professor, Department of Conservative Dentistry & Endodontics, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh, India

[2]Junior Resident, Department of Conservative Dentistry & Endodontics, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh, India

[3]Professor & Head, Department of Conservative Dentistry & Endodontics, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh, India

[4]Assistant Professor, Department of Conservative Dentistry & Endodontics, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh, India

[5]Junior Resident, Department of Conservative Dentistry & Endodontics, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh, India

[6]Consultant Endodontist, Lucknow, Uttar Pradesh, India.

*Corresponding Author:

Dr. Tanu Tewari

Associate Professor, Department of Conservative Dentistry & Endodontics, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh, India

Email ID: tanutewari@gmail.com

ABSTRACT

Introduction: Irrigation and disinfection of root canals plays a very important role in the success of a root canal treatment as the root canal system anatomy is extremely complex and diverse and it is not always possible to clean and shape it effectively.

Aim: The aim of this study was to evaluate and compare the antimicrobial efficacy of chlorhexidine, neem leaf extract, lemon peel extract and combination of neem leaf extract & lemon peel extract against ***Enterococcus faecalis***.

Method: The microorganisms selected for this study is ***Enterococcus faecalis*** *(*ATCC 29212). The culture was grown overnight in Brain Heart Infusion Broth at 37 degree Celsius on a rotary shaker at 150 revolutions per minute, with bacterial growth monitored by changes in turbidity at 24 hours. Spread plate method was used to determine the bacterial count in CFU at different time intervals. After confirming the growth of the respective microorganisms in the broth, paper points were dipped in test tube containing test microorganism and then transferred to test tube containing test irrigants for stipulated time and then incubated for 48 hrs.

After incubation broth of each test tube was spread on nutrient agar plate of 10^{-6} dilution. NAM plates were then inverted for bacterial count.

Results

Chlorhexidine showed maximum antimicrobial activity against ***E. faecalis***, followed by Neem leaf extract, Lemon peel extract and lastly mixture of Neem leaf and Lemon peel extract, difference being statistically significant.

Conclusion: CHX exhibited the most potent antibacterial activity, on other hand, among the tested herbal agents, Neem leaf extract showed comparable efficacy at prolonged exposure times, suggesting its potential as an alternative root canal irrigant. Lemon peel extract also displayed antimicrobial properties, though its effectiveness was lower compared to CHX and Neem leaf extract.

Keywords: ***Enterococcus faecalis***, Chlorhexidine, Neem leaf extract, Lemon peel extract, Root Canal Irrigant, Root Canal Irrigation.

INTRODUCTION

Successful root canal treatment requires effective cleaning and shaping through disinfection and three dimensional obturation to inhibit bacterial recolonization and facilitate healing of periradicular tissues.[1]

Root canal infections are polymicrobial in nature. This typically consists of aerobic and anaerobic bacteria, virus and fungi.[2]

Enterococcus faecalis is most commonly recovered from infected root canals of failed endodontic therapy and canals with persistent infections. It can survive in extreme environmental conditions such as low pH, high salinity and high temperature. It is also capable of thriving in poor nutrient environment. It generally invades and metabolize within the dentinal fluids and binds to collagen fibres.[3]

In order to eliminate ***E. faecalis*** from root canal space, numerous measures have been described, including the use of various instrumentation techniques, irrigation regimens and intracanal medicaments.[4]

Chemo-mechanical preparation of the infected root canal reduces the bacterial load. However, presence of isthmus and lateral canals in apical one-third of root canal system remain infected having bacterial load due to inaccessibility.[5] Hence, irrigation is the most effective way to disinfect these inaccessible areas of the root canal system, that are not touched by mechanical instrumentation.[6]

Several chemical irrigants are commonly used for root canal disinfection.[7]

Chlorhexidine is a well-known and widely used antimicrobial agent with broad spectrum activity. It exhibits bacteriostatic properties at low concentrations (0.02% to 0.06%) and bactericidal effects at high concentrations (2%). Chlorhexidine has the property of substantivity. [8,9]

To improve the effectiveness of root canal irrigation against ***E.faecalis*** new alternative irrigants have been investigated.

Use of neem (Azadirachta indica) as an endodontic irrigant might be advantageous because it is a biocompatible antioxidant and thus not likely to cause any adverse reactions.[10]

Use of neem as an endodontic irrigant might be advantageous because it is a biocompatible antioxidant and thus not likely to cause any adverse reactions. A study using neem leaf extract has shown that it is a viable medicament against C. albicans, E. faecalis and even mixed culture when compared to sodium hypochlorite. [11]

Neem has shown biocompatibility with root canal periapical tissues, minimizing the potential for cytotoxic effects linked to synthetic irrigants.[10]

Lemon is an important medicinal plant. Citrus flavonoids have a broad spectrum of biological activity and antimicrobial activity of the peel extract is directly concerned with the components that they contain.[12]

Citrus peel is made up from limonene (90%) and citral (5%). Limonene, a biologically active compound responsible for antimicrobial property is present in the essential oil of the citrus peel which has potential application in food systems as natural preservative.[13]

Researchers are focusing on herbal extracts as endodontic irrigants as they have antibacterial properties and comparatively fewer harmful effects.[14]

Not many studies have been done on these herbal extracts especially on lemon as an endodontic irrigant.

Therefore, the present study has been undertaken to comparatively evaluate antimicrobial efficacy of selected herbal extracts in comparison with conventional irrigants.

Methodology:

Test Organism- The test microorganism used in this study was ***Enterococcus faecalis*** (ATCC 29212)

Test Irrigants- The irrigants evaluated in this study were 2% Chlorhexidine, Neem Leaf Extract, Lemon peel Extract Mixture of Neem Leaf Extract and Lemon peel Extract.

Method for growth of E. faecalis strain

The microorganisms selected for this study was ***Enterococcus faecalis*** (ATCC 29212). The culture was grown overnight in Brain Heart Infusion Broth at 37 degrees Celsius on a rotary shaker at 150 revolutions per minute, with bacterial growth monitored by changes in turbidity at 24 hours.

A pure culture of ***Enterococcus faecalis*** (ATCC 29212) was spectrophotometrically adjusted to an optical density of 560 nm, correlating to the turbidity of the McFarland 0.5 scale and then was cultured on Mueller Hinton Agar.

Preparation of Irrigants –

Neem Leaf extract and Lemon Peel Extract

For the preparation of each irrigant, Neem leaf extract and Lemon Peel extract, the dried sample powder (5.0 gm) was weighed, placed in a cheese cloth and kept in thimble; Methanol solvent used for extraction. Solvent was added to a round bottom flask, which was attached to a Soxhlet extractor and condenser. The dried sample was loaded into the thimble, placed inside the Soxhlet extractor. The solvent was heated using the heating mantle. The condensate then drips into the reservoir containing the thimble. Once the level of solvent reached the siphon it was poured back into the flask and the cycle begins again. The process ran for a total of 6 hours.

The collected extract was concentrated using a rotary evaporator to dryness and then weighed.
Methodology:
For the test 80 sterile paper points were dipped in E. faecalis culture broth, then immersed each paper point into LE of concentration 1mg / ml, NE & LE+NE of concentration 1mg / ml & chlorhexidine solution of concentration 0.2 % for. 5 min, 10 min, 15 min, 1 hr and 24 hr individually in a test tube in triplicate .A tube was considered as positive control (paper point dipped in E. faecalis culture broth for different time intervals, then immersed into 100 ppm ciprofloxacin solution for 1 hr and dipped in 2 ml broth) and one another tube was considered as a negative control (paper point dipped in E. faecalis culture broth for 5 mins and dipped in 2 ml broth). After this step immersed paper points were dipped in 2 ml broth and incubated for 48 hrs. After 48 hours incubated broth of each required time condition optical density was recorded for each tube using UV-Visible Double Beam Spectrophotometer (PC-Based Double Beam UV-VIS Spectrophotometer 2201) and broth of each tube was spreaded on a nutrient agar plate of 10^{-6} dilution.

The spread plate of 10^{-6}dilution of only E. faecalis culture was considered as negative control and the spread plate of 10^{-6} dilution of E. faecalis culture and 100ppm was considered as negative control. Prepared media with defined compositions were sterilized by autoclaving (at 121°C, 15 psi, 15-20 min) and then cooled and molten media was used. 0.1 ml of sample's desired dilution was transferred to blank sterilized petri plates and cooled and molten media was poured onto the sample. It was mixed thoroughly by swirling and allowed to set. Then the NAM plates were inverted for 48-72 hours for bacterial count. Plates with between 25 and 250 colonies were counted. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC).
Expression of results: The result is expressed as the number of colonies formed per gram of the undiluted sample.
Total colonies count per ml of sample = colonies count x dilution factor/amount of sample plated

RESULT:
All data obtained from the microbiological evaluation were tabulated and statistically analyzed using Statistical Package for the Social Sciences (SPSS-21) software (IBM Corp., Armonk, NY, USA). Quantitative variables, including bacterial colony-forming units (CFU/mL) and optical density (OD) values measured at 560 nm, were expressed as mean ± standard deviation (SD) for each experimental irrigant group at all time intervals.
The study involved four experimental irrigant groups: 2% Chlorhexidine (2% CHX), Neem Leaf extract (1mg/ml) (NE), Lemon Peel Extract(1mg/ml) (LE), and a combination of Neem Leaf extract(1mg/ml) and Lemon Peel Extract (1mg/ml) (NE+LE). Antibacterial efficacy was evaluated at multiple time points (5 minutes, 10 minutes, 15 minutes, 1 hour, and 24 hours).
At each time interval, one-way analysis of variance (ANOVA) was used to compare mean CFU counts and mean OD values among the four irrigant groups.
Table 1 reports the Mean ± SD bacterial counts (CFU/mL) following irrigation with experimental irrigants at different time intervals.
At 5 minutes
CFU counts were comparable across all groups, with no statistically significant differences.2% CHX = NE (1mg/ml) = LE(1mg/ml) = NE(1mg/ml) +LE(1mg/ml)
At 10 minutes
NE (1mg/ml) showed the lowest CFU count, followed by 2% CHX. NE (1mg/ml) +LE (1mg/ml) and LE (1mg/ml) showed higher residual bacterial counts, with LE(1mg/ml) being the least effective. NE(1mg/ml) < 2% CHX < NE(1mg/ml) +LE(1mg/ml) < LE(1mg/ml)
At 15 minutes
2% CHX, NE (1mg/ml), and LE (1mg/ml) achieved complete bacterial elimination, whereas NE (1mg/ml) + LE (1mg/ml) showed residual bacterial growth.
2% CHX = NE (1mg/ml) = LE (1mg/ml) < NE (1mg/ml) +LE (1mg/ml)
At 1 hour
All irrigant groups demonstrated complete bacterial elimination with no detectable CFU. 2% CHX = NE (1mg/ml) = LE (1mg/ml) = NE (1mg/ml)+LE (1mg/ml)
At 24 hours
Sustained antibacterial effect was observed in all groups, with no bacterial regrowth.
2% CHX = NE (1mg/ml) = LE (1mg/ml) = NE (1mg/ml) +LE (1mg/ml)

Group	5 min (Mean ± SD)	10 min (Mean ± SD)	15 min (Mean ± SD)	1 hr (Mean ± SD)	24 hr (Mean ± SD)
2% CHX	4.0×10^{-8} ± 2.83×10^{-8}	2.0×10^{-8} ± 1.41×10^{-8}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NE(1mg/ml)	6.0×10^{-8} ± 4.24×10^{-8}	1.0×10^{-8} ± 0.71×10^{-8}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
LE (1mg/ml)	6.0×10^{-8} ± 4.24×10^{-8}	4.0×10^{-8} ± 2.83×10^{-8}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NE(1mg/ml) +LE (1mg/ml)	5.0×10^{-8} ± 3.54×10^{-8}	3.0×10^{-8} ± 2.12×10^{-8}	1.0×10^{-8} ± 0.71×10^{-8}	0.0 ± 0.0	0.0 ± 0.0
p VALUE	0.734	<0.001	<0.001	-	-

Table 1: Bacterial Growth (CFU/mL) of Broth After Irrigation With Experimental Irrigants at Different Time Intervals (Mean ± SD)

Optical Density (OD) values provide a measure of bacterial regrowth in broth. At 5 and 10 minutes, 2% CHX showed the lowest turbidity, followed by NE (1mg/ml), NE (1mg/ml)+LE(1mg/ml) and LE (1mg/ml). At 15 minutes, 2% CHX maintained significantly lower OD values relative to NE (1mg/ml), LE (1mg/ml), and NE (1mg/ml) +LE (1mg/ml). At 1 hour, 2% CHX continued to outperform all other irrigants. At 24 hours, 2% CHX reached the lowest OD, far below other irrigants confirming enduring antimicrobial dominance.

All time intervals showed significant differences ($p < 0.001$), highlighting the consistent superiority of 2% CHX in inhibiting bacterial regrowth.

Group	5 min (Mean ± SD)	10 min (Mean ± SD)	15 min (Mean ± SD)	1 hr (Mean ± SD)	24 hr (Mean ± SD)
2% CHX	0.634 ± 0.064	0.351 ± 0.041	0.301 ± 0.015	0.113 ± 0.004	0.069 ± 0.003
NE (1mg/ml)	0.728 ± 0.044	0.536 ± 0.028	0.465 ± 0.045	0.366 ± 0.006	0.250 ± 0.030
LE (1mg/ml)	0.937 ± 0.034	0.647 ± 0.090	0.531 ± 0.022	0.431 ± 0.056	0.312 ± 0.027

NE+LE (1mg/ml)	0.769 ± 0.024	0.718 ± 0.050	0.542 ± 0.065	0.423 ± 0.046	0.308 ± 0.03
pVALUE	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2: Mean Absorbance (OD at 560 nm) of broth after irrigation with experimental irrigants at different time intervals following 48-hour incubation

DISCUSSION

In root canal, ***Enterococcus faecalis*** dominates primary lesions and is also persistent in periradicular lesions even after root canal treatment as ***E. faecalis*** has the ability to survive extreme environment and high pH. [15]

The bactericidal effect of Chlorhexidine (CHX) is due to the cationic molecule binding to extra-microbial complexes and negatively charged microbial cell walls, thereby altering the osmotic equilibrium of the cells. At low concentrations, it has bacteriostatic effect. At higher concentrations, CHX has a bactericidal effect.[16]

Despite the individual antimicrobial potential of Neem leaf extract and Lemon peel extract, their efficacy as endodontic irrigants remains largely unexplored. This study aimed to evaluate and compare the antimicrobial effectiveness of Neem leaf extract, Lemon peel extract and mixture of Neem leaf and Lemon peel extract against ***E. faecalis.***

According to Okmen G et al, among the three solvents (ethanol, methanol and water), ethanol and methanol were observed to be efficient at extracting the maximum number of secondary metabolites.[17]

The present study used a direct contact test to evaluate the antimicrobial efficacy of 2% chlorhexidine (CHX), Neem leaf extract, Lemon peel extract and mixture of Neem leaf and Lemon peel extract against ***Enterococcus faecalis*** (ATCC 29212).

Chlorhexidine (CHX): The CFU analysis for CHX revealed a rapid, two-phase bactericidal action. A statistically significant reduction in viable bacterial counts occurred between 5 and 10 minutes, followed by complete microbial elimination (0.0 CFU/mL) by the 15-minute interval. This pattern confirms CHX's swift and definitive killing efficacy, with no bacterial recovery detected thereafter.

Neem Leaf Extract (NE): Neem Leaf extract demonstrated the largest and highly significant reduction in CFU between 5 and 10 minutes. This potent early activity culminated in complete bacterial eradication by 15 minutes, indicating that NE possesses a rapid mechanism of antimicrobial action that rivals the speed of the gold-standard CHX in this model.

Lemon Peel Extract (LE): While a significant decrease in CFU was observed from 5 to 10 minutes, the magnitude of this reduction was smaller than that seen with NE. However, with continued exposure, LE achieved complete bacterial elimination by the 15-minute mark, suggesting its mechanism requires slightly longer contact time to reach maximum bactericidal effect.

Neem Leaf+Lemon Peel Extract Combination (NE+LE): Contrary to expectations, the combination extract displayed the slowest and most progressive reduction in viable counts. It was the only group where bacterial presence remained detectable at 15 minutes, requiring a full hour of exposure to achieve sterility. This delayed kinetics suggests a potential antagonistic interaction between the two natural extracts, making the combination less effective in the short term than either component alone.

In a study by Mustafa M. (2016), the antimicrobial efficacy of neem has been compared with that of the chlorhexidine gluconate and NaOCl, and it was found that efficacy of neem was comparable to that of other commonly used gold standard compounds.[18]

This is in accordance with findings of this study that the potent early activity of Neem leaf extract culminated in complete bacterial eradication by 15 minutes, indicating that it possesses a rapid mechanism of antimicrobial action that rivals the speed of the gold-standard CHx in this study.

Some studies revealed that the substantivity of CHX for longer period like Khandani et al found that 5 min treatment with 2% CHX solution induced substantivity upto 4weeks. Rosenthal et al indicated that substantivity of CHX was extended for up to 12 weeks.[19]

This is in accordance with result of this study as the change from 1 to 24 hours was minimal and non-significant, indicating CHx had reached a very low, stable plateau of inhibition, showing sustained antimicrobial activity or substantivity.

Dhanavade ***et al***. suggest that different alcoholic extracts of lemon peel give antimicrobial activity against different bacterial isolates better than the aqueous extract.[20] which is in accordance with the preparation of

ethanolic extract of lemon peel in this study.

LIMITATIONS OF THE RESEARCH

The in-vitro nature of the study means the findings might not fully translate to clinical settings. Using a single bacterial strain may not accurately reflect the complex oral microbiota and does not take into consideration of other bacteria associated with endodontic infections.

CONCLUSION:

CHX exhibited the most potent antibacterial activity, on other hand, among the tested herbal agents, Neem leaf extract showed comparable efficacy at prolonged exposure times, suggesting its potential as an alternative root canal irrigant. Lemon peel extract also displayed antimicrobial properties, though its effectiveness was lower compared to CHX and Neem leaf extract.

REFERENCES:

1 Narayanan LL, Vaishnavi C. Endodontic microbiology. J Conserv Dent. 2010 Oct;13(4):233-9

2 Tronstad L, Sunde PI. The evolving new understanding of endodontic infections. Endod Topics 2003;6:57-77

3 Irankhah S, Soudi MR, Gharavi S. Ex situ study of Enterococcus faecalis survival in the recreational waters of the southern coast of the Caspian Sea. Iran J Microbiol. 2016 Apr;8(2):101-7.

4 J, Manoil D, Näsman P, Belibasakis GN, Neelakantan P. Microbiological Aspects of Root Canal Infections and Disinfection Strategies: An Update Review on the Current Knowledge and Challenges. Front Oral Health. 2021 Jun 25;2:672887

5 J.F.Siqueria Jr. Aetiology of root canal treatment failure: why well-treated teeth can fail. Int Endod J. 2001 Jan;34(1):1-10.

6 Dioguardi M, Gioia GD, Illuzzi G, Laneve E, Cocco A, Troiano G. Endodontic irrigants: Different methods to improve efficacy and related problems. Eur J Dent. 2018 Jul-Sep;12(3):459-466

7 Iqbal A. Antimicrobial irrigants in the endodontic therapy. Int J Health Sci (Qassim). 2012 Jun;6(2):186-92

8 Kumar H (2013) An in vitro evaluation of the antimicrobial efficacy of Curcuma longa, Tachspermum ammi, "Chlorhexidine" gluconate and calcium hydroxide on "E. Faecalis". J Conserv Dent 16: 144-147.

9 Martina LP, Ebenezar AV, Ghani MF, Narayanan A, Sundaram M, Mohan AG. An in vitro comparative antibacterial study of different concentrations of green tea extracts and 2% chlorhexidine on Enterococcus faecalis. Saudi Endodontic Journal. 2013 Sep 1;3(3):120-4.

10 Kennedy J, Hussey D. The antimicrobial effects of root canal irrigation and medication. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2007 Apr 1;103(4):560

11 Ambareen Z, Konde S, Raj SN, Kumar NC. Antimicrobial efficacy of herbal extracts. Int J Oral Health Dent. 2015 Jul;1(3):108-3.

12 Hindi NK, Chabuck ZA. Antimicrobial activity of different aqueous lemon extracts. Journal of Applied Pharmaceutical Science. 2013 Jun 27;3(6):074-8.

13 Rai MR, Chawla R, Mirza A. Study on different extraction methods and antimicrobial potential of citrus peel and leaf. International Journal of Current Microbiology and Applied Sciences. 2020;9(11):246-54.

14 Jose J, Shoba K, Aman S, Tomy N, Christie S. Comparative Evaluation of Antimicrobial Activity of Green Tea Extract, Garlic Extract, Neem Leaf Extract and Sodium Hypochlorite as Root Canal Irrigants against E. Faecalis and C. albicans. An In Vitro Study. ***Int J Curr Microbiol Appl Sci***. 2015;4:384-91.

15 Shubham S, Samant PS, Khanal R, Gautam V, Birring OJ, Arora C, Mishra R, Kandel L. Papaya extract as new endodontic irrigant. ***IOSR J Dent Med Sci.*** 2018;17:23-8.

16 Gomes BPFA, Aveiro E, Kishen A. Irrigants and irrigation activation systems in Endodontics. Braz Dent J. 2023 Jul-Aug;34(4):1-33.

17 Ökmen G, Arslan K, Tekin RC. The antimicrobial activity of Citrus limon L. against foodborne pathogens and its anti-oxidant and antibiofilm properties. I***nt. J. Ayur. Med.*** 2021, Vol 12 (2), 301-308

18 Mustafa M Antibacterial Efficacy of Neem (Azadirachta indica) Extract against Enterococcus faecalis: An in vitro Study. J Contemp Dent Pract. 2016

19 Mahendra A, Koul M, Upadhyay V, Dwivedi R. Comparative evaluation of antimicrobial substantivity of different concentrations of chlorhexidine as a root canal irrigant: An in vitro study. J Oral Biol Craniofac Res. 2014 Sep-Dec;4(3):181-5.

20 Dhanavade MJ, Jalkute CB, Ghosh JS and Sonawane KD. Study Antimicrobial Activity of Lemon (Citrus lemon L.) Peel Extract Br. J. Pharmacol. Toxicol 2011; 2(3): 119-122