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In-vitro evaluation of antibacterial efficacy of piper betel leaf extract and *Morinda citrifolia* incorporated in root canal sealer against *Enterococcus faecalis*

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Abstract
Aim: To evaluate and compare *in vitro* the antibacterial efficacy of Bioceramic root canal sealer modified with Piper betel leaf and *Morinda citrifolia* against *Enterococcus faecalis*.
Materials and mathods: A Bioceramic root canal sealer was mixed with two herbal extracts (Piper betel leaf, *Morinda citrifolia*) which was divided into two groups. Their antibacterial efficacy was evaluated against standard strains of *Enterococcus faecalis* at various time intervals using Agar Diffusion Test. The mean zones of inhibition were measured. The statistical analysis was performed using the SPSS software. Analysis of the data between groups was carried out using paired and unpaired t test.
Results: Both groups demonstrated a reduction in the zone of inhibition over time. However, Group 2 (BIO-C sealer + *Morinda citrifolia*) consistently exhibited a larger zone of inhibition than Group 1 (BIO-C sealer + Piper betel leaf) at each time interval. With p-values all below 0.01, the differences between the groups across the time points are statistically significant.
Conclusion: The study demonstrated that both groups exhibited significant antimicrobial activity against *Enterococcus faecalis*, with a reduction in the zone of inhibition over time. However, Group 2 (BIO-C sealer + *Morinda citrifolia*) consistently showed a larger zone of inhibition compared to Group 1 (BIO-C sealer + Piper betel leaf), indicating superior antibacterial efficacy. These findings suggest that *Morinda citrifolia*, when combined with BIO-C sealer, may be more effective as an antimicrobial agent in root canal treatment than Piper betel leaf. This supports the potential use of medicinal plant extracts as alternatives to conventional antimicrobial agents in endodontics.

Keywords: *Enterococcus faecalis*, Bioceramic root canal sealer, Piper betel leaf, *Morinda citrifolia*, zone of inhibition

Introduction

Microbes are considered as the primary etiological agents in endodontic diseases. One of the factors in determining the success of endodontic treatment is sealing root canals with materials possessing potent bactericidal effect. Numerous studies report a high success rate for endodontic treatment, ranging from 85% to 95%, with failure rates between 0.6% and 4.4%[1]. Bacterial persistence after root canal irrigation using effective intracanal irrigants can be attributed to the complex anatomy of the root canal, including accessory canals, isthmuses, and fines [2, 3]. Therefore, it is crucial to effectively obturate the root canal space and establish a three-dimensional seal, preventing any infection caused by persistent bacteria and ultimately eliminating them [4].

Despite Gutta-percha being the primary material used for filling the root canal, it is insufficient on its own to completely fill the canal space or stick to the canal walls [3]. Clinically, using an endodontic sealer with antibacterial properties would be beneficial for eliminating any remaining bacteria and preventing bacterial infiltration into the root canal space following three dimensional obturation [4, 3].

Enterococcus faecalis, a gram-positive facultative anaerobe is found in 4 to 40% of initial endodontic infections and in 24 to 77% of recurring endodontic infections [5]. This species exhibits the highest level of resistance among oral cavity bacteria and is considered as the possible cause of failure of root canal treatment [6, 7].

Currently, Herbal medications have gained attention and are utilized by approximately 80% of the global population for health-related purposes [8, 9]. Due to cytotoxic reactions of sealers and their inability to eliminate bacteria completely from dentinal tubules, trend to use natural plant extracts have been introduced.

Piper betle L., a perennial plant native to Asia and Southeast Asia, is recognized for its heart-shaped leaves [10]. It belongs to the Piperaceae family, which includes a wide variety of plant species widely grown and found in countries like India, Sri Lanka, and Bangladesh [11]. Piper betel is highly valued in the pharmaceutical industry due to its reputed medicinal properties, including aromatic, digestive, expectorant, euphoria-inducing, stimulant, antibacterial, antiprotozoal, carminative, aphrodisiac, and antifungal effects [12]. M. citrifolia, commonly referred to as Noni, is a herb renowned for its diverse medicinal properties. Traditionally used for centuries, it is valued for its antifungal, antiviral, antibacterial, antihelminthic, analgesic, and hypotensive effects. Two of its key antibacterial compounds, alizarin and L-asperuloside, have demonstrated greater potency than 2% CHX [13].

Several *in-vitro* experiments have examined the antibacterial properties of herbal extracts combined with a root canal sealer to combat oral anaerobic bacteria. These studies have concluded that the greatest inhibition zone was observed [6, 14, 15, 16]. Although numerous studies have investigated the effectiveness of herbal extracts against oral anaerobic bacteria, limited attention has been given to the utilization of bioceramic sealer mixed with piper betel leaf and *Morinda citrifolia*.

Hence, the present *in-vitro* study aims to evaluate and compare the antibacterial effects of piper betel leaf and *Morinda citrifolia* when incorporated into a bio-ceramic sealer against *Enterococcus faecalis*.

Aim of the study

To evaluate and compare *in vitro* the antibacterial efficacy of bioceramic root canal sealer modified with Piper betel leaf and *Morinda citrifolia* against *Enterococcus faecalis*.

Objectives of the study

- To evaluate *in vitro* the antibacterial efficacy of Piper betel leaf mixed with Bioactive RCS against *Enterococcus faecalis*.
- To evaluate *in vitro* the antibacterial efficacy of *Morinda citrifolia* mixed with Bioactive RCS against *Enterococcus faecalis*.

Materials and method

Armamentarium and material

- Sodium hypochlorite
- Saline
- Bioactive RCS

For microbiological processing

- Culture media plates
- Brain heart infusion broth
- Whatman filter paper (No. 1)
- Whatman filter paper (No. 4)
- McFarland Scale
- Mueller Hinton agar plates
- Sterile loops
- Test tubes
- Incubator
- Methanol
- Airtight containers
- Endodontic millimeter ruler

Antimicrobial agents

- Piper betel leaf extract
- *Morinda citrifolia*

Methodology

Standard strains of *Enterococcus faecalis* was obtained from Father Muller Medical College, Mangalore. The herbal extracts were obtained from KVG Ayurveda College, Sullia. Bioceramic Root canal sealer was used. Agar diffusion test was conducted. The study was conducted at K.V.G Dental College & Hospital, Sullia, D.K and evaluation of antimicrobial efficacy was done at Microbiology Department of Father Muller Medical College, Mangalore. The collection, storage, sterilization, handling of the antimicrobial agents and microbial samples was followed according to Occupational Safety & Health Administration (OSHA) and the Centre for Disease Control & Prevention recommendations and guidelines.

Sample preparation

Preparation of herbal extracts

The present *in-vitro* study was conducted to evaluate the antimicrobial efficacy of Bioceramic root canal sealer mixed with two herbal extracts Piper betel leaf and *Morinda citrifolia* against *Enterococcus faecalis*, at various time intervals using the agar diffusion test.

Methanolic extracts of Piper betel leaf and *Morinda citrifolia* was utilized in the study.

Preparation of piper betel leaf extract

Fresh leaves of Piper betle (about 1 kg) was washed twice thoroughly with tap water to emancipate dirt, after which they were allowed to dry under direct sunlight. Once dried, they were ground into powder form by mechanical blender, which were then stored in air-tight bottles until further use. The fine powder (50 g) was macerated in 1 L of methanol for a period of seven days at room temperature with occasional stirring. After seven days, Whatman filter paper (No. 1) was used to filter the solutions. The extracts in crude form was obtained and dried at room temperature, followed by weighing and stored them in sterile vials in the refrigerator.

Preparation of *Morinda citrifolia*

Dried M.citrifolia fruit (MC) was grinded and macerated with 99% methanol and water. MC (5 g) was continuously extracted with 99% methanol until exhaustion using soxhlet apparatus. The extract was filtered and evaporated to be dried under a vacuum. MC (100 g) was continuously macerated with DI water until exhaustion, and then Whatman No. 4 filter paper was used to filter the extract. The water extracts were lyophilized, dried and stored at -20 °C until being used to reduce the probability of degradation of the active compounds.

Preparation of microbial strains and growth conditions

Enterococcus faecalis strains (ATCC 29212) was obtained from Microbiology Department of Father Muller Medical College, Mangalore. The samples were cultured on BHI broth at 37^{o}C and the McFarland scale was adjusted to 0.5

Preparation of the inoculums

A sterilized loop of 4mm diameter was used to pick up four to five colonies. These colonies were dissolved in a test tube containing 5 mL of 0.85% saline solution, resulting in a turbidity level of 0.5 on McFarland scale, which corresponds to a concentration of 10^8 colony-forming units per milliliter. For the inoculation process, Petri dishes with a diameter of 90 mm and a thickness of 4mm, containing Mueller-Hinton agar was utilized.

To ensure an even distribution of the inoculums, the bacterial dilution was evenly swabbed onto freshly prepared agar plates using the “lawn technique”. Each plate was divided into two equal sections. Within each section, wells with diameter of 6 mm and depth of 5 mm were created using previously formulated copper wells. Finally, the wells in each section were filled with a mixture of bioceramic root canal sealer and two different herbal extracts.

Incubation

In order to facilitate the even spread of the sealer mixed with herbal extracts through the agar, the plates containing the inoculated mixture were left at room temperature for a duration of two hours. The MH agar plates were then incubated at a temperature of 37°C.

The size of the inhibition zone was recorded at 24 hours, 48 hours, 72 hours, and lastly 7 days.

Reading the size of zone of inhibition

Evidence of the growth of inhibitory zones around each sealer combined with a herbal extract was observed as a lack of bacterial colonization (clearing of agar) near the sealer-herbal extract mixture.

Using an endodontic millimeter ruler, the most consistent diameter of the inhibition zone was measured, and the 6 mm diameter of the well was subtracted from this measurement as the cut-off value.

Measurements exceeding this value indicated a significant inhibition of bacterial growth. Broader inhibition zones demonstrated a higher level of antimicrobial activity of the bioceramic sealer mixed with herbal extracts.

The collected data was statistically analyzed by comparing the average sizes of the inhibition zones for each sealer.

Sample grouping

Two groups were established by combining the sealer with herbal extracts. To evaluate their antibacterial effectiveness against *Enterococcus faecalis*, 0.1ml of each modified sealer was employed and their antibacterial efficacy was compared.

The study consisted of two groups:

- **Group 1 (n=8):** Bioactive Root canal sealer mixed with Piper betel leaf extract.
- **Group 2 (n=8):** Bioactive Root canal sealer mixed with *Morinda citrifolia*

Results

Table 1: Intra group comparison of zone of inhibition (in mm) among 2 groups at 3 different time intervals

Group 1: BIO-C sealer +Piper betel leaf				
	24 Hrs	48 Hrs	7 Days	p value
N	8	8	8	<0.01*
Mean± Std. Deviation	9.20±0.22	6.08±0.44	2.47±0.66	
Minimum	8.89	5.45	1.50	
Maximum	9.57	6.78	3.20	

*ANOVA test was used and p value <0.05 was considered statistically significant

Group 2: BIO-C sealer+*Morinda citrifolia*				
	24HRS	48HRS	7 DAYS	p value
N	8	8	8	<0.01*
Mean± Std. Deviation	12.87±0.74	9.35±0.19	5.59±0.28	
Minimum	12.02	9.10	9.65	
Maximum	13.85	9.65	5.92	

*ANOVA test was used and p value <0.05 was considered statistically significant

Table 2: Inter group comparison of zone of inhibition (in mm) between 2 groups at 3 different time intervals

Time interval	Group 1: BIO-C sealer +Piper betel leaf	Group 2: BIO-C sealer+Morindacitrifolia	p value
24 HRS	9.20	12.87	<0.01*
48 HRS	6.08	9.35	
7 Days	2.47	5.59	

*ANOVA test was used and p value <0.05 was considered statistically significant

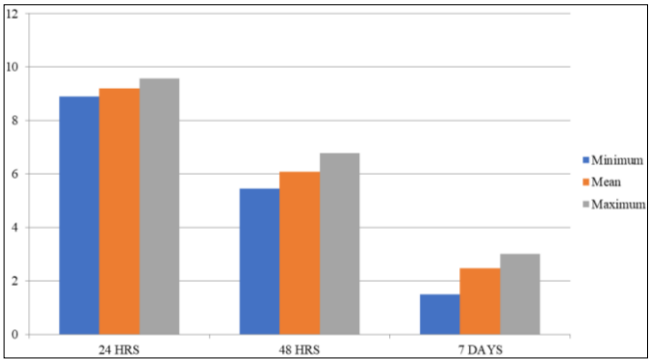

Fig 1: Zone of inhibition (in mm) of BIO-C sealer +Piper betel leaf at 3 different time intervals

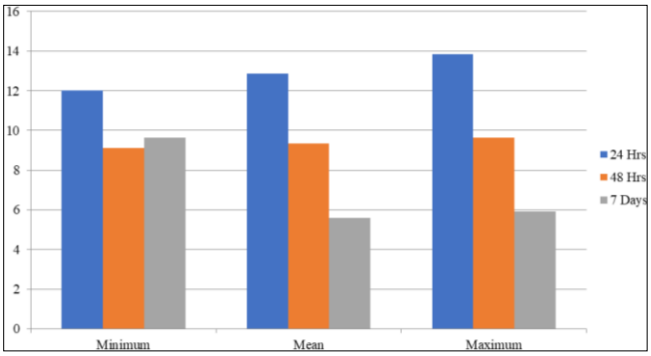

Fig 2: Zone of inhibition (in mm) of BIO-C sealer + *Morinda citrifolia* at 3 different time intervals

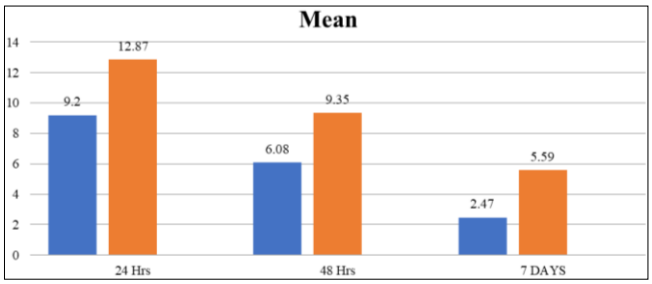

Fig 3: Comparison of Zone of inhibition (in mm) between 2 groups at 3 different time interval

Both the groups showed decrease in the zone of inhibition over time. Group 2 (BIO-C sealer + *Morinda citrifolia*) consistently showed a larger zone of inhibition compared to Group 1 (BIO-C sealer + Piper betel leaf) at all time intervals. The p-values are all < 0.01, indicating statistically significant differences between the groups across time intervals

Discussion

Elimination of bacteria by cleaning, shaping, and obturation of the root canal system is the objective of root canal treatment. Bioceramic (BC) sealers are known to possess biological activity. It is highly radiopaque and hydrophilic which forms hydroxyapatite on setting and chemically bonds to both dentin and gutta-percha points. It is antibacterial during setting due to its highly alkaline pH and exhibits absolutely zero shrinkage.

Hypersensitivity, immune suppression, and allergic reactions are some of the adverse effects associated with antimicrobial agents, and the continuous evolution of bacterial resistance has necessitated the search for medicinal plant alternatives as antimicrobial agents. Medicinal plants

are rich sources of bioactive compounds such as alkaloids, flavonoids, and phenolic compounds. Methanolic extracts were shown to have a greater activity compared to ethanolic or aqueous extracts because more phytoconstituents are leached out from it when compared to ethanolic extracts. Hence, methanolic extracts of herbal plants were prepared as test medicaments in this study.

Enterococcus faecalis (a gram-positive coccus) was chosen as the test organism because it is a facultative anaerobe that is the most resistant species in the oral cavity and the possible cause of failure of root canal treatment. There are insufficient scientific reports that indicate the antimicrobial activities of herbal extracts mixed with endodontic root canal sealers. Hence, the present study evaluated the antimicrobial efficacy of bioceramic sealer mixed with herbal extracts.

The present study found significant antimicrobial activity of Bioceramic sealer against the test microorganism. The antibacterial effect of the Bioceramic sealer may be due to the combination of high pH and active calcium hydroxide diffusion. The results revealed that Group 2 (BIO-C sealer + *Morinda citrifolia*) exhibited more effective antibacterial activity with a larger zone of inhibition over time compared to Group 1 (BIO-C sealer + Piper betel leaf). These findings are consistent with similar studies by Bathula vimala chaitanya, et. Al in 2016 [17] NaOCl (3%) which showed maximum antibacterial activity against E. faecalis, followed by *Morinda citrifolia* and turmeric extracts. A study by Emily Mesa Jamelarin, *et al.* in 2019 [18] on Antibacterial tests against E. faecalis, E. coli, P. aeruginosa and S. aureus showed that ethanolic and methanolic leaf extracts of P. betel had the highest zone of inhibition against E. coli and S. aureus, respectively. Also previous study by Francisca Lívia Parente Viana, *et al.* in 2021 [19], revealed that Bioceramic sealers shows greater antimicrobial activity than resin-based sealers when in direct contact with biofilms ($P < 0.05$). This suggests that *Morinda citrifolia* may have superior antibacterial properties in combination with BIO-C sealer compared to Piper betel leaf.

Conclusion

Within the limitations of the study, better antimicrobial activity of BC sealer was seen in eradication of E. faecalis.

Bio-C sealer mixed with methanolic extract of *Morinda citrifolia* produced larger inhibitory zones than Bio-C sealer mixed with Piper betel leaf. Bioactive compounds present in the herbal extracts used in this study are potent antimicrobial agents that are beneficial in the field of dentistry in combating dental diseases.

The extracts of Piper betel leaf & *Morinda citrifolia* mixed with Bio-C sealer enhanced the antimicrobial properties by decreasing the cytotoxicity of conventional sealers by their additive effect of the combination.

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