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Original Article

Dentistry Section

Comparative Evaluation of Bacterial Microleakage in Furcal Perforation Repair Materials Modified with Eggshell Powder: An In-vitro Study

SAROJ REDDY PAWAR[1], ANAMIKA CHETAN BORKAR[2], SANKET ARAS[3], DIPTI JITENDRA KHARAJANI[4], VRUSHALI SHRIGANESH PADWAL[5]

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ABSTRACT

Introduction: Furcal perforation is a clinically significant endodontic complication that may compromise the root canal-periodontal interface and adversely affect tooth prognosis. Mineral Trioxide Aggregate (MTA) and Biodentine™ are widely used calcium silicate-based repair materials, although further improvement in their sealing ability remains desirable. Eggshell Powder (ESP) is a calcium-rich natural biomaterial that may enhance interfacial adaptation and resistance to bacterial penetration.

Aim: To evaluate and compare the bacterial microleakage of MTA and Biodentine™ modified with 3% and 5% by weight of ESP for furcal perforation repair.

Materials and Methods: This in-vitro study was conducted in the Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Pimpri, Pune, India, from September 2025 to February 2026. A total of 80 extracted mandibular molars were used. Among them, 60 specimens were allocated to six experimental groups (n=10): unmodified MTA, MTA modified with 3% or 5% ESP, unmodified Biodentine™, and Biodentine™ modified with 3% or 5% ESP. Ten teeth with unrepaired perforations and ten teeth with intact furcations served as positive and negative controls, respectively. Standardised 4-mm furcal perforations were prepared and assessed using a dual-chamber *Enterococcus faecalis* leakage model over 50 days. Within-material comparisons were performed using the Fisher-Freeman-Halton exact test, followed by pairwise Fisher's-exact tests with Bonferroni correction.

Results: Leakage occurred in 80%, 20%, and 50% of the control, 3% ESP-modified, and 5% ESP-modified MTA groups, respectively, with a significant overall difference among the MTA groups (p=0.034). Corresponding leakage in the Biodentine™ groups was 70%, 10%, and 20%, with a significant overall difference (p=0.023). The lowest leakage was observed with 3% ESP-modified Biodentine™. However, none of the individual pairwise comparisons remained statistically significant after Bonferroni correction.

Conclusion: ESP modification was associated with significant overall differences in bacterial leakage within both MTA and Biodentine™ groups. The 3% ESP-modified formulations showed the lowest observed leakage, particularly 3% ESP-modified Biodentine™. However, the absence of statistically significant pairwise differences after multiplicity adjustment warrants cautious interpretation. Further adequately powered physicochemical, biological, and clinical studies are required before clinical application can be recommended.

Keywords: Dental cements, Dental leakage, *Enterococcus faecalis*, Furcation defects, Root canal filling materials

INTRODUCTION

Furcal perforations are a significant complication in clinical endodontics, as they disrupt the integrity of the root canal system and the periodontium, often leading to inflammatory destruction of the supporting tissues [1]. The long-term prognosis of a tooth with a furcation injury is highly dependent on the clinician's ability to achieve a hermetic seal that prevents bacterial ingress and subsequent inflammatory responses in the furcal area. MTA has long been recognised as a gold-standard material for such repairs due to its superior sealing ability and biocompatibility. However, the search for modifications that can further enhance its physical properties and biological performance, while remaining cost-effective, continues to be a primary focus in dental biomaterials research [2].

Bioceramic-based materials, such as Biodentine™ and MTA, function by releasing calcium ions that facilitate the formation of a mineralised barrier. Despite their clinical success, these materials can exhibit prolonged setting times or higher costs, prompting interest in natural, calcium-rich alternatives for modification [3]. ESP, a common agricultural byproduct, is composed primarily of calcium carbonate and has been investigated for its potential to serve as a natural source of hydroxyapatite or a filler in dental cements. Its high calcium content makes it a promising additive for enhancing the mineralising potential of endodontic repair materials [4].

The success of any furcal repair material is evaluated through its microleakage resistance, which is typically assessed using bacterial or dye leakage models. Inadequate sealing at the material-dentin interface allows for microbial colonisation, which is the primary cause of endodontic treatment failure. Therefore, evaluating how natural modifications like ESP influence the sealing capacity of established materials like MTA and Biodentine™ is essential for developing next-generation reparative agents [5]. Despite the extensive documentation of MTA and Biodentine™ as effective repair materials, there is a distinct paucity of literature regarding the specific effects of incorporating varying concentrations of natural ESP on their bacterial microleakage resistance in furcal perforations [6]. Most existing studies focus on standard formulations without exploring bio-waste modifications that could potentially improve both biological and economic outcomes [7-9]. The present study is necessary to determine if ESP-modified cements can maintain or exceed the sealing performance of traditional materials. Consequently, this in-vitro study aimed to comparatively evaluate the bacterial microleakage of furcal perforation repair materials (MTA and Biodentine™) when modified with 3% and 5% ESP [5]. The current study explores the integration of sustainable, natural materials into clinical practice. These findings attempt to provide critical insights into how cost-effective modifications can be utilised without compromising the technical excellence required for complex

endodontic procedures, potentially offering a more accessible approach to high-quality tooth-saving treatments.

MATERIALS AND METHODS

This in-vitro study was conducted in the Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Pimpri, Pune, India, from September 2025 to February 2026. The study protocol was approved by the Institutional Ethics Committee of Dr. D. Y. Patil Dental College and Hospital, Pimpri, Pune (DYPDCH/IEC/82/2024). The study was reported in accordance with the Checklist for Reporting In-vitro Studies (CRIS) in dentistry guideline [10].

Inclusion criteria:

- Freshly extracted permanent mandibular first and second molars;
- Teeth with an intact furcation area, free of furcal caries or perforations;
- Non fused, separate mesial and distal roots;
- Teeth free of prior endodontic treatment.

Exclusion criteria:

- Teeth with furcal caries, furcal cracks, or furcal pathology;
- Teeth with internal or external root resorption;
- Teeth with pre-existing furcal perforations;
- Previously root canal treated teeth;
- Teeth with developmental anomalies of root morphology.

Sample size calculation: Sample size was estimated using G*Power software, version 3.1.9.4, for an omnibus Chi-square test comparing bacterial leakage across six experimental groups. The calculation was informed by the bacterial leakage methodology reported by Francis T et al., who evaluated MTA-Angelus and Biodentine™ in a dual-chamber *Enterococcus faecalis* leakage model over 50 days [11]. Assuming a large effect size of Cohen's w=0.50, a two-sided alpha level of 0.05, 80% statistical power, and five degrees of freedom, the minimum required sample size was 52 specimens. The sample was increased to 60 specimens to permit equal allocation of 10 specimens to each of the six experimental groups.

A total of 80 extracted mandibular molars were used: 60 specimens were allocated to six experimental groups, and 20 additional specimens were assigned to positive and negative control groups. The sample size of ten specimens per group is consistent with comparable in-vitro bacterial microleakage investigations reported in the literature. Given the controlled, single-outcome binary nature of this in-vitro experiment (leakage versus no leakage) and the inclusion of both positive and negative controls to validate the model, a group size of n=10 was considered adequate for the purpose of the present study.

Study Procedure

Eggshell Powder (ESP) preparation and characterisation: ESP in the present study denotes a chemically converted hydroxyapatite derivative of eggshell, not native calcium carbonate powder. The ESP was prepared through sequential chemical and thermal processing [Table/Fig-1]. Chicken eggshells were cleaned with sterile distilled water, boiled at 100°C for 10 minutes to remove the inner membrane, dried, and ground using a mortar and pestle. The sieved powder was calcined at 1200°C to ensure pathogen elimination. The calcined powder was dissolved in concentrated nitric acid; pH was adjusted to 9.5 with ammonium hydroxide (1:1), and 1 M citric acid was added. Dropwise addition of 1 M diammonium hydrogen phosphate (1 mL/min) yielded a white precipitate, which was redissolved, stirred at 70°C for two hours to form a transparent gel, heated at 250°C to yield a black precursor, and sintered at 900 °C for two hours to produce the final white ESP [12]. Particle size (150–250 nm) and zeta potential were confirmed using a zeta potential analyser.



[Table/Fig-1]: Sequential preparation of Eggshell Powder (ESP): a) Boiling of eggshells; b) cleaned and crushed eggshells; c) grinding into fine particles; d) sieving of the powder after calcination in a muffle furnace; e) dissolution in nitric acid; f) addition of diammonium hydrogen phosphate; g) formation of a white precipitate; h) development of a transparent gel; i) heating of the gel at 250°C; j) formation of a black precursor; and k) sintering at 900°C for 2 hours to obtain the final white Eggshell Powder (ESP).

Preparation of modified repair materials and experimental groups: The ESP was incorporated into MTA and Biodentine™ at 3% and 5% concentrations [Table/Fig-2]. ESP was incorporated into MTA Angelus and Biodentine™ at 3% and 5% Weight-to-weight (w/w) concentrations relative to the dry powder component of each material [13], weighed on a calibrated digital analytical balance (Shimadzu). Modified powders were blended uniformly and mixed per manufacturer instructions. The 60 specimens were allocated as shown in [Table/Fig-3].



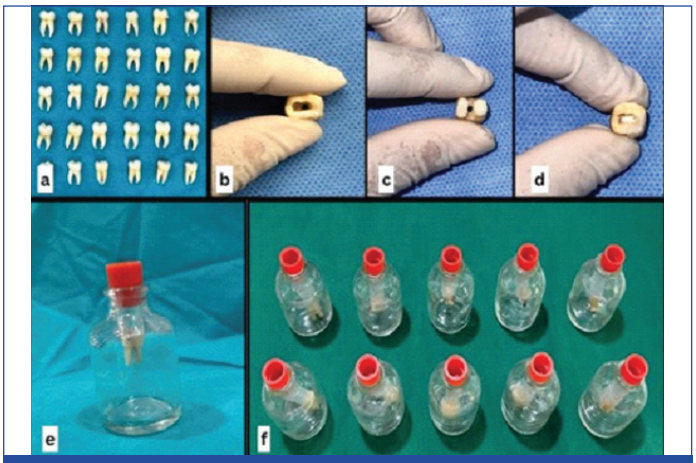
[Table/Fig-2]: Eggshell Powder (ESP) modified with 3% and 5% of Biodentine™ and MTA.

Groups	Materials	Description	n
Group-1	MTA Angelus	Unmodified MTA Angelus (control)	10
Group-2	MTA+3% ESP	MTA Angelus modified with 3% ESP by weight	10
Group-3	MTA+5% ESP	MTA Angelus modified with 5% ESP by weight	10
Group-4	Biodentine™	Unmodified Biodentine™ (control)	10
Group-5	Biodentine™+ 3% ESP	Biodentine™ modified with 3% ESP by weight	10
Group-6	Biodentine™+ 5% ESP	Biodentine™ modified with 5% ESP by weight	10
Group-7	Positive control	Open (unrepaired) furcal perforation	10
Group-8	Negative control	Intact furcation sealed with sticky wax (no perforation)	10

[Table/Fig-3]: Group-wise allocation of specimens.

Perforation repair and setting: Repair materials were delivered to the perforation site using an endodontic carrier gun and condensed with a plugger. A moist cotton pellet was placed in the pulp chamber, and specimens were wrapped in moist sterile gauze. All specimens were incubated at 37°C and 100% relative humidity for 24 hours to allow material setting prior to assembly of the bacterial leakage apparatus [14]. It is acknowledged that full-integrity setting of MTA Angelus ideally requires 72 hours [15]; the 24-hour setting period is noted as a limitation of the current protocol.

Bacterial microleakage assessment: A dual-chambered bacterial leakage model adapted from Francis T et al., (2019) was employed [11]. The upper chamber comprised a 3 cm sterile PVC pipe into which each specimen was mounted with the furcal repair site exposed; the tooth-pipe interface was sealed with sticky wax. The upper chamber was inserted into a 10 mL sterile glass vial (lower chamber) containing 5 mL of sterile Brain Heart Infusion (BHI) broth. The complete assembly was pre-incubated at 37°C and 100% humidity for 72 hours to confirm sterility before bacterial challenge. Specimen preparation and bacterial leakage assessment are illustrated in [Table/Fig-4].



[Table/Fig-4]: Steps in specimen preparation and bacterial microleakage assessment: (a) Extracted mandibular molar specimens; (b,c) Standardised 4 mm furcal perforation; (d) Perforation repaired with the assigned test material; (e) Dual-chamber bacterial leakage model; and (f) Ten specimens included in each experimental group.

A standardised suspension of Enterococcus faecalis (PTCC 1778; 9×10^8 CFU/mL) was prepared by turbidimetric standardisation. The stock suspension was diluted 1:900 with sterile Brain Heart Infusion broth to obtain a final inoculating concentration of approximately 1×10^6 CFU/mL. One hundred microlitres of this suspension was introduced into the pulp chamber every 48 hours throughout the 50-day observation period. Prior to each reinoculation, the existing inoculum was aspirated and plated on MacConkey agar to confirm bacterial viability. The lower chamber BHI broth was inspected daily by a single blinded examiner (masked to group allocation) for turbidity and colour change (clear to turbid) indicative of bacterial passage [11,16]. Turbidity was microbiologically confirmed by subculture on MacConkey agar at 37°C for 24 hours. Following the appearance of turbidity in the lower chamber, a sample from the broth was streaked onto agar plates and incubated at 37°C for 24 hours to confirm the growth of *Enterococcus faecalis*. The day on which turbidity was first observed and microbiologically confirmed was recorded as the leakage date for each specimen. The positive control was used to confirm that the experimental model could detect bacterial leakage when the perforation was left open. The negative control was used to confirm that no leakage occurred when the furcation remained intact and was completely sealed.

STATISTICAL ANALYSIS

Data were analysed using IBM Statistical Package for Social Sciences (SPSS) Statistics version 25.0 (IBM Corp., Armonk, NY, USA) [17]. Bacterial microleakage at day 50 was treated as a binary categorical outcome and expressed as frequency and percentage of leaked and non leaked specimens. To evaluate the effect of ESP modification within each repair material, separate comparisons were performed among the control MTA, 3% ESP-modified MTA, and 5% ESP-modified MTA groups and among the control Biodentine™, 3% ESP-modified Biodentine™, and 5% ESP-modified Biodentine™ groups. Owing to the small sample size and expected cell frequencies below five, the Fisher-Freeman-Halton exact test was used for each overall three-group comparison. Pairwise comparisons were subsequently performed using Fisher's-exact test. A Bonferroni-adjusted significance threshold of $p<0.0167$ was applied to the three pairwise comparisons within each material category, while $p<0.05$ was considered statistically significant for the overall comparisons. Cumulative leakage across the 5-day observation intervals was summarised descriptively using frequencies, percentages, tables, and a line graph.

RESULTS

Bacterial microleakage was assessed in 60 experimental specimens (groups 1-6, n=10 per group) over a 50-day observation period using a dual-chambered *E. faecalis* leakage model. Model validity was confirmed by the positive control group (group 7: open perforation), in which all 10 specimens (100%) demonstrated turbidity within the first five days, and the negative control group (group 8: intact furcation sealed with sticky wax), in which zero specimens leaked throughout the study period.

Leakage incidence at day 50: the bacterial microleakage for all experimental groups at the end of the 50-day observation period, including the number of leaked and non leaked specimens, the overall leakage percentage, and the interval of first detected leakage, is summarised in [Table/Fig-5]. At day 50, control groups exhibited the highest leakage: MTA at 80% (8/10) and Biodentine™ at 70% (7/10), with first events starting at days 21-25. ESP modification reduced leakage in all experimental groups. For MTA, 3% ESP (20%) performed better than 5% ESP (50%). Biodentine™ with 3% ESP showed the lowest overall leakage (10%), while 5% ESP showed 20%. Notably, both 3% ESP modifications delayed initial leakage until the final 46th - 50th day interval.

Groups	Leaked (n)	Not leaked (n)	Leakage (%)	First leakage interval
Group 1- Control MTA	8	2	80.0	21st-25th day
Group 2- MTA+3% ESP	2	8	20.0	46th-50th day
Group 3- MTA+5% ESP	5	5	50.0	36th-40th day
Group 4- Control Biodentine™	7	3	70.0	21st-25th day
Group 5- Biodentine™+3% ESP	1	9	10.0	46th-50th day
Group 6- Biodentine™+5% ESP	2	8	20.0	41st-45th day
Group 7- Positive control	10	0	100.0	1st-5th day
Group 8- Negative control	0	10	0.0	N/A

[Table/Fig-5]: Microleakage for all experimental groups at the end of the 50-day observation period.

Temporal pattern of cumulative bacterial leakage: The cumulative number of leaked specimens recorded at each 5-day interval across the 50-day study period is presented in [Table/Fig-6]. All groups remained leakage-free for the first 20 days. Initial leakage occurred at days 21-25 in control MTA (final: 8/10) and control Biodentine™ (final: 7/10). ESP-modified groups exhibited delayed onset: MTA+5% ESP at days 36-40 (final: 5/10) and Biodentine™+5% ESP at days 41-45 (final: 2/10). The 3% ESP-modified groups (MTA and Biodentine™) showed the greatest resistance, with no leakage detected until the final 46-50-day interval, resulting in cumulative totals of 2/10 and 1/10, respectively. Cumulative bacterial leakage patterns across 50 days are shown as a line graph in [Table/Fig-7].

S. No.	Interval	Group 1- control MTA	Group 2- MTA+3% ESP	Group 3- MTA+5% ESP	Group 4- control Biodentine™	Group 5- Biodentine™+3% ESP	Group 6- Biodentine™+5% ESP
1	1-5 days	0	0	0	0	0	0
2	6-10 days	0	0	0	0	0	0
3	11-15 days	0	0	0	0	0	0
4	16-20 days	0	0	0	0	0	0
5	21-25 days	1 (10%)	0	0	2 (20%)	0	0
6	26-30 days	2 (20%)	0	0	3 (30%)	0	0
7	31-35 days	2 (20%)	0	0	4 (40%)	0	0
8	36-40 days	4 (40%)	0	1 (10%)	4 (40%)	0	0
9	41-45 days	5 (50%)	0	3 (30%)	4 (40%)	0	2 (20%)
10	46-50 days	8 (80%)	2 (20%)	5 (50%)	7 (70%)	1 (10%)	2 (20%)

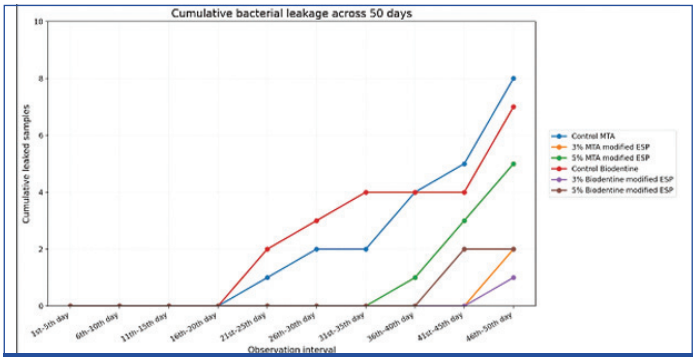
[Table/Fig-6]: Cumulative number of leaked specimens recorded at each 5-day interval across the 50-day study period.

Material category	Groups	Leaked, n (%)	Not leaked, n (%)	Overall exact p-value
MTA	Control MTA	8 (80.0)	2 (20.0)	0.034
	MTA+3% ESP	2 (20.0)	8 (80.0)	
	MTA+5% ESP	5 (50.0)	5 (50.0)	
Biodentine™	Control Biodentine™	7 (70.0)	3 (30.0)	0.023
	Biodentine™+3% ESP	1 (10.0)	9 (90.0)	
	Biodentine™+5% ESP	2 (20.0)	8 (80.0)	

[Table/Fig-8]: Within-material comparison of bacterial leakage at day 50.

Final leakage status at day 50: Final bacterial leakage occurred in 25/60 specimens (41.7%), while 35 (58.3%) remained leakage-free. Group 1 (8/10) and group 4 (7/10) showed the highest leakage, followed by group 3 (5/10). Groups 2, 5, and 6 recorded the lowest counts (2/10, 1/10, and 2/10, respectively), demonstrating the effectiveness of ESP modification over control groups.

Comparison among MTA groups: At day 50, bacterial leakage was observed in eight of 10 specimens (80.0%) in the control MTA group, two of 10 specimens (20.0%) in the 3% ESP-modified MTA group, and five of 10 specimens (50.0%) in the 5% ESP-modified MTA group. The overall difference in leakage status among the three MTA groups was statistically significant using the Fisher-Freeman-Halton exact test (p=0.034) [Table/Fig-8].



[Table/Fig-7]: Line graph showing cumulative bacterial leakage across 50 days.

Pairwise comparison showed lower leakage in the 3% ESP-modified MTA group than in the control MTA group (20.0% versus 80.0%; p=0.023). However, this difference did not meet the Bonferroni-adjusted significance threshold of p<0.0167. No statistically significant differences were observed between the control MTA and 5% ESP-modified MTA groups (p=0.350) or between the 3% and 5% ESP-modified MTA groups (p=0.350).

Comparison among Biodentine™ groups: Bacterial leakage was observed in seven of 10 specimens (70.0%) in the control Biodentine™ group, one of 10 specimens (10.0%) in the 3% ESP-modified Biodentine™ group, and two of 10 specimens (20.0%) in the 5% ESP-modified Biodentine™ group. The overall difference among the three Biodentine™ groups was statistically significant using the Fisher–Freeman–Halton exact test (p=0.023) [Table/Fig-8].

Pairwise comparison showed lower leakage in the 3% ESP-modified Biodentine™ group than in the control Biodentine™ group (10.0% versus 70.0%; p=0.020). The difference between the control Biodentine™ and 5% ESP-modified Biodentine™ groups was not statistically significant (p=0.070), and no significant difference was observed between the 3% and 5% ESP-modified Biodentine™ groups (p=1.000). None of the pairwise comparisons met the Bonferroni-adjusted significance threshold of p<0.0167.

DISCUSSION

The management of furcal perforations remains a clinical challenge, requiring materials that provide a durable seal against microbial challenges. The present study investigated the impact of modifying MTA and Biodentine™ with ESP, a natural calcium-rich biomaterial, evaluating their sealing ability against *Enterococcus faecalis* [18]. The most significant finding was that 3% ESP modification markedly enhanced the resistance of both materials to bacterial microleakage. Specifically, 3% ESP-modified Biodentine™ demonstrated the most superior performance, with only 10% leakage and the most delayed onset of bacterial penetration over 50 days. This improvement is likely due to the high calcium carbonate content of ESP, which promotes interfacial mineral deposition and improves adaptation at the dentin-material interface.

These findings align with and extend established literature regarding calcium silicate-based repair materials. Francis T et al., (2019) confirmed that both MTA and Biodentine™ are effective for perforation repair, though they found no statistically significant difference between them in their specific 50-day leakage model [11]. However, the descriptive superiority of Biodentine™ observed in the current study is consistent with reports by Mohan D et al., who noted that Biodentine™ exhibited lower dye penetration than MTA [19]. While Nikoloudaki GE et al., (2014) previously reported MTA as having the lowest microleakage, such discrepancies are often attributed to variations in experimental models [20]. The use of a bacterial leakage model in the present study provides a more stringent, clinically relevant challenge compared to simpler dye penetration tests.

The incorporation of ESP as a bioactive modifier is strongly supported by recent physicochemical research. Shetty AK et al., (2020) characterised ESP as having high calcium levels and an amorphous nature favourable for quick calcium release and apatite formation [8]. Beshr KA and Abdelrahim RA (2019) further established that adding ESP to MTA and Biodentine™ enhances mineral deposition and sealing by forming a robust interfacial layer [7]. Interestingly, the concentration-dependent effect noted

in the present study - where 3% ESP modification outperformed 5% ESP - parallels the findings of Salem A et al., (2023), who observed that lower concentrations of ESP improved compressive strength and setting time [9]. This indicates an "optimal threshold" where the bio-additive enhances bioactivity without disrupting the material's homogeneity or increasing porosity.

Furthermore, Abraham S et al., (2023) previously demonstrated that modifying glass ionomer cement with 7% ESP improved its sealing ability, reinforcing the potential of this natural byproduct [21]. As suggested by Batra D et al., (2025) and Makhlouf M et al., (2020), calcium silicate-based materials provide the most favourable biological and sealing profile for treating iatrogenic mishaps [22,23]. Subhi H et al., (2025) noted that Biodentine™ possesses intrinsic antibacterial activity against *E. faecalis*, which, when enriched by a calcium-rich modifier like ESP, creates a barrier against persistent endodontic infections [24]. Consequently, 3% ESP modification represents a significant step toward optimising cost-effective, high-performance endodontic biomaterials.

Separate within-material analyses demonstrated significant overall differences in bacterial leakage among the three MTA groups and among the three Biodentine™ groups. The greatest numerical reduction in leakage was observed with 3% ESP modification. However, the individual pairwise comparisons did not remain statistically significant after Bonferroni adjustment, indicating that these findings should be interpreted cautiously in view of the limited sample size.

The clinical implications of the present study are noteworthy for the field of dentistry. By utilising ESP - an abundant, low-cost, and naturally derived byproduct - practitioners can potentially enhance the performance of expensive bioceramics like Biodentine™ and MTA. The finding that 3% ESP-modified Biodentine™ provides a superior seal suggests a promising pathway toward more effective and accessible perforation repair protocols. This modification not only improves the success rate of treating procedural mishaps but also aligns with the growing dental trend of utilising bioactive, natural additives to promote periradicular healing and tissue regeneration.

Limitation(s)

Despite these promising results, several limitations must be acknowledged. As an in-vitro study, it could not fully replicate the dynamic complexity of the oral environment, such as occlusal loading, salivary flow, or the host immune response. The study utilised a mono-microbial challenge with *E. faecalis*, whereas clinical infections are typically polymicrobial. Furthermore, while microleakage was assessed, complementary analyses such as Scanning Electron Microscopy (SEM) for interfacial characterisation or push-out bond strength testing would have provided a more comprehensive mechanical and structural profile of the modified materials.

CONCLUSION(S)

Within the limitations of this in-vitro study, the modification of MTA and Biodentine™ with ESP significantly improves their sealing ability against bacterial microleakage. A 3% weight modification appears to be the optimal concentration, with 3% ESP-modified Biodentine™ demonstrating the highest resistance to penetration by *Enterococcus faecalis*. These results suggest that ESP is a valuable, cost-effective bioactive additive that can enhance the clinical performance of existing endodontic biomaterials. While these findings are promising, further in vivo research is recommended to validate these results under clinical conditions.

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PARTICULARS OF CONTRIBUTORS:
1. Postgraduate Student, Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India.
2. Professor, Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India.
3. Lecturer, Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India.
4. Postgraduate Student, Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India.
5. Postgraduate Student, Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Anamika Chetan Borkar,
Professor, Department of Conservative Dentistry and Endodontics, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pimpri,
Pune-411018, Maharashtra, India.
E-mail: borkar.anamika@gmail.com

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