ORIGINAL RESEARCH

A Novel Combination of Zinc Oxide with Two Essential Oils Exerts Antimicrobial Effect against Endodontic Pathogens *In Vitro*

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Abstract

Aims and objectives: Evaluating the antimicrobial efficacy of the novel combinations of zinc oxide mixed with ajwain oil (ZNOA) and combination of ajwain and eugenol (ZNOAE) vs conventionally used zinc oxide eugenol (ZNOE) against endodontic pathogens like *Escherichia coli* and *Enterococcus faecalis*.

Materials and methods: The pure cultures of *E. coli* (MTCC 443) and *E. faecalis* (MTCC 439) were revived and grown on selective cultural media. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the test materials were determined correspondingly through sequential dilution and agar well diffusion methods, as per Clinical and Laboratory Standard Institute (CLSI) guidelines. The data values were presented as mean ± standard deviation (SD). The comparisons among groups were completed through the one-way analysis of variance (ANOVA) test, whereas intragroup pairwise comparisons were completed using the unpaired *t*-test ($p < 0.05$).

Results: Minimum inhibitory concentration values against *E. coli* and *E. faecalis* of ZNOE were 250 and 500 µg/mL, ZNOA was 250 µg/mL, and ZNOAE were 125 and 250 µg/mL, correspondingly. MBC values in the form of inhibition zone against *E. coli* by ZNOE were 21.33 ± 1.53 mm, ZNOA 18.67 ± 1.53 mm, and ZNOAE 20.33 ± 1.53 mm. The *E. faecalis* inhibition zone for ZNOE was 14.33 ± 2.08 mm, ZNOA 18.67 ± 2.08 mm, and ZNOAE 24.33 ± 1.53 mm.

Conclusion: All test materials demonstrated good antibacterial efficacy. However, between the novel combinations of test materials, ZNOA showed better antimicrobial efficacy against resistant endodontic pathogens than ZNOE.

Keywords: Antibacterial agents, Culture media, Pulpectomy, Root canal filling materials, Tooth deciduous.

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Introduction

Polymicrobial natures of endodontic infections predominantly consist of obligate anaerobic bacteria. Certain bacterial species, such as *Fusobacterium nucleatum*, *Propionibacterium* spp., *Enterococcus faecalis*, *Escherichia coli*, *Parvimonas micra*, and *Eubacterium* spp. persist even after comprehensive intracanal disinfection and are responsible for the failure of endodontic therapies.[1] The complex, tortuous ribbon-shaped configurations of the deciduous tooth root canals further reduce the potency of chemomechanical and biomechanical cleansing, warranting the complete elimination of the vegetative and nonvegetative forms of the microorganisms.[2] Hence, to eliminate the residual microorganisms from deciduous root canals, obturating materials with optimal antibacterial properties are preferred.[3]

The traditional and most commonly used obturating material for deciduous root canals is zinc oxide eugenol (ZNOE),[4] demonstrating numerous advantages like easy handling, anti-inflammatory and analgesic properties, cost-effectiveness, and antimicrobial effect. Though embracing considerable benefits, the limited antibacterial efficacy against certain bacterial species like *E. faecalis*, *E. coli*, *Pseudomonas aeruginosa*, *Porphyromonas gingivalis*, and *Prevotella intermedia* restricts its utility.[5,6] To overcome this disadvantage, few researchers attempted to modify ZNOE by adding peppermint oil, tea tree oil, and/or thyme oil with mutable success rates.[7,8] One such essential oil, ajwain oil, has been evaluated extensively in the field of Ayurveda. It is extracted from *Trachyspermum ammi* seeds and contains thymol (40–50%). Other ingredients contained include β-pinene, γ-terpinene, carvacrol,[9] p-cymene, and α-pinene, exhibiting numerous medicinal attributes such as analgesia, reduced inflammation, hepatoprotective as well as germicidal activity.[10] It demonstrates antibacterial efficacy against various strains of bacteria like *Staphylococcus epidermidis*, *E. coli*, *E. faecalis*, *P. aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*.[11] However, its use in dentistry has not been explored sufficiently. The study found promising antibacterial efficacy of ajwain oil against several microbial types accountable for endodontic infections.[12] Similarly, Gilani et al. found a reduction in inflammation in rat models when ajwain oil was used.[9,13]

Given the medicinal properties of ajwain oil and the quest to search for new materials with better antimicrobial properties, two

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Table 1: Test materials

Group	*Test materials*
Group I (control)	ZNOE
Group II (test material)	ZNOA
Group III (test material)	ZNOAE

novel combinations of ajwain oil, that is, ajwain oil individually and in combination with eugenol (1:1 ratio) admixed with zinc oxide respectively, have been evaluated in the present study against two resistant endodontic pathogens namely *E. coli* and *E. faecalis*.

Materials and Methods

The experiment was piloted in the Pediatric and Preventive Dentistry Department after getting ethical clearance from the IEC (MUHS/PG-T/E1/2208/2018. 02/08/2018). Table 1 shows all the materials used in the experiment.

The compositional details of the test materials are mentioned here:

- Zinc oxide powder (PRIME Dental Products Pvt. Ltd., Thane, Maharashtra, India).
- Eugenol (Healthvit DF Pharmacy Gota, Ahmedabad, Gujarat, India).
- Ajwain oil (Deepti Products, Ratnagiri, Maharashtra, India).

Microorganisms Used

The bacterial species *E. coli* (MTCC 443) and *E. faecalis* (MTCC 439) were acquired from Institute of Microbial Technology, Chandigarh, India, after approval from the IEC.

Culture Media Used

The stock cultures of bacteria were used. Muller Hinton Agar (HiMedia Laboratories Private Limited, Mumbai, India) is the most widely used growing medium for detecting the vulnerability of *E. coli* and *E. faecalis*.[14] Brain heart infusion (BHI) broth (HiMedia Laboratories Private Limited, Mumbai, India) was used for cultivating test microbes.[7,15]

Standardization and Preparation of Test Materials

A total of 200 mg zinc oxide powder was dispensed on a disinfected glass slab. The powder and eugenol (0.07 mL) were mixed with a sterile stainless steel spatula to a creamy consistency.[16] The remaining test materials were also prepared as the previous one due to the unavailability of ingredient standardization.[16] The test materials were made ready before starting the microbiological experiment.[17,18]

Preparation of Stock Solution

Based on recommendations of Clinical and Laboratory Standard Institute (CLSI) guidelines, the stock solutions were prepared from 1000 µg/mL.[19] The freshly prepared zinc oxide paste was vortexed with 10 ml sterile distilled water (MC Dalal and Co. Chennai, India) to achieve homogeneous solutions of 1000 µg/mL concentration. The same procedure was carried out for zinc oxide mixed with ajwain oil (ZNOA) and combination of ajwain and eugenol (ZNOAE). The light-proof containers were used to preserve all the stock solutions, preventing their desiccation and oxidation.

Preparation of Inocula

Revival and Subculturing of Bacterial Strains

The lyophilized bacterial vials of both *E. coli* and *E. faecalis* were added to BHI broth for their revival at 37°C in biosafety cabinet II, under the aseptic conditions.[18] After revival, bacterial cells of the log phase were again suspended in BHI broth to make secondary bacterial aliquots, with a cell count of approximately 1×10^7 CFU/mL at 0.5 McFarland standards at OD_{600}:0.6–0.7 (optical density).[19] This secondary culture was used to determine minimum inhibitory concentration (MIC) by means of serial dilution as well as minimum bactericidal concentration (MBC) by well diffusion technique.

Determining Minimum Inhibitory Concentration of Test Materials by Serial Dilution Method

A stock solution of 1000 µg/mL was prepared. A total of 1 mL of each test material's stock solution was diluted doubly from 1000 to 1.95 µg/mL. The last broth tube without any material was marked a negative control. Following CLSI guidelines, the MIC of all the test materials was determined.[19] A total of 5 µL of secondary bacterial aliquot were vortexed in each MIC tube (MC Dalal and Co. Chennai, India) to make a homogenous suspension. All the test tubes were incubated in the microbiological incubator at 37°C for 24–48 hours to accomplish bacteria growth. Each test material's MIC was inspected by spectrophotometry at OD_{600}: 0.6–0.7 optical density to identify broth turbidity. The lowermost concentration of test material displaying no bacterial growth was labeled as MIC of the material for the corresponding bacterium. All the processes were carried out in triplicates to abate errors.[20]

Determining Minimum Bactericidal Concentration of Test Materials Using Agar Well Diffusion Method

The lawn culture of each trial bacterium was done on a Muller Hinton agar plate. A total of 50 µL of bacterial aliquots from inoculums were evenly streaked on culture plates with sterile swabs to attain uniform bacterial lawn culture.[21,22] In each plate, wells of six mm diameter were cut at equal intervals and loaded with freshly mixed test materials. Each mixed material was weighed precisely at a quantity of 50 µg and added to pinched wells of respective culture media, streaked with the lawn culture of each test bacteria. The culture plates were incubated at 37°C for 24 hours. Petri plates were observed for inhibition zones in the form of a "clear halo." They were measured using an antibiotic zone scale from the center of the well to the clearest periphery.[17,18] The tests were repeated three times to minimize errors for both test microbes.[20]

Statistical Analysis

The quantitative data for the MIC and MBC values were tabulated in MS Excel and expressed as mean ± standard deviation (SD). The data were analyzed using descriptive statistics with Statistical Package for the Social Sciences for Windows, Version 22.0 (IBM Corp., Armonk, New York, United States of America). A one-way analysis of variance (ANOVA) test was used for intergroup comparison of the MIC and MBC, while between-group pairwise comparisons were computed using the unpaired *t*-test. The probability value "*p*" was set at 95%, where $p \geq 0.05$ was considered nonsignificant (NS), $p \leq 0.05$ was considered statistically significant (S), and $p \leq 0.001$ was labeled highly significant (HS).

Results

The MIC and MBC values obtained by triplicating the procedure were calculated in the form of mean and SDs and presented in Figures 1 and 2.

Figure 1 illustrates the MIC of test materials for test bacteria. The results showed that *E. coli* was inhibited by ZNOAE at 125 µg/mL

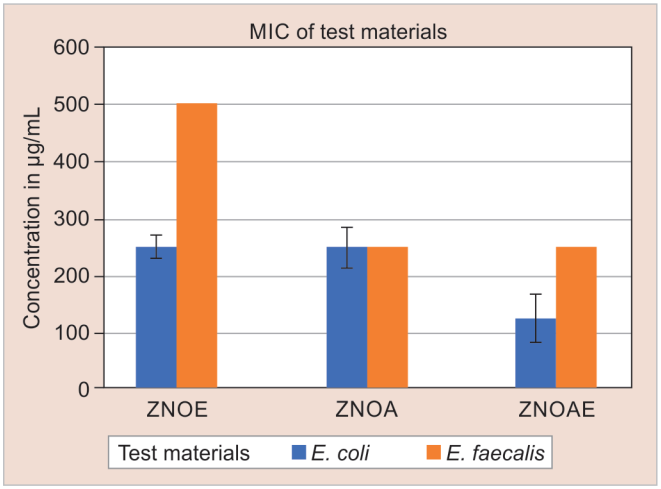

Fig. 1: Minimum inhibitory concentration of the test materials against test bacteria

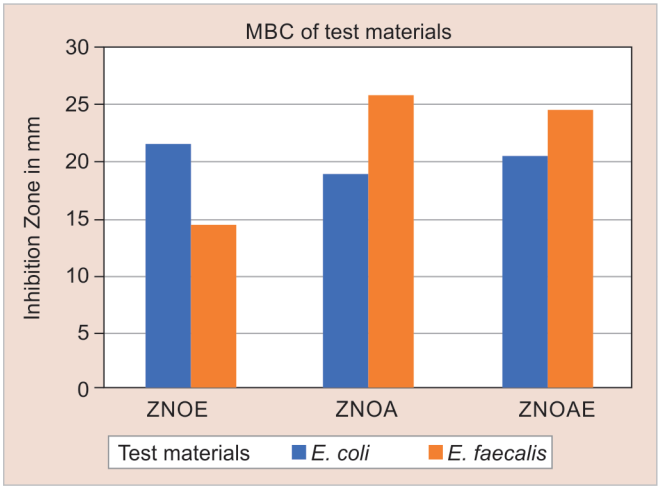

Fig. 2: Minimum bactericidal concentration of the test materials against test bacteria

Table 2: Criteria for determination of the antibacterial activity of the test materials

Rank	*Inhibition zone (diameter in mm)*	*Interpretation*
No	0	Resistant
Weak	≤0.1–11.5	Weakly sensitive
Medium	11.5–19.7	Moderately sensitive
Strong	>19.7	Highly sensitive

Table 3: Intergroup comparison of MIC of test materials against *E. coli* and *E. faecalis*

Group		*N*	*Mean*	*SD*	*t*	*p*	*Inference*
ZNOE	*E. coli*	3	250.00	0.0	–	–	NS
	E. faecalis	3	500.00	0.0			
ZNOA	*E. coli*	3	250.00	0.0	–	–	NS
	E. faecalis	3	250.00	0.0			
ZNOAE	*E. coli*	3	125.00	0.0	–	–	NS
	E. faecalis	3	250.00	0.0			

Where; *t*, unpaired *t*-test; *p*, probability value; NS, nonsignificant ($p > 0.05$); S, significant ($p < 0.05$); HS, highly significant ($p < 0.001$)

and ZNOE and ZNOA at 250 µg/mL. In comparison, *E. faecalis* was inhibited by ZNOA and ZNOAE at 250 µg/mL concentration and ZNOE at 500 µg/mL dose.

The MBC of the test materials was determined based on the criteria in Table 2, as the inhibition zone (clear halo) around the well punched in the agar medium following the ranking scheme.[6,12] The larger inhibition zone indicated higher antibacterial activity.

The results of the MBC for the test materials against test bacteria are shown in Figure 2. For *E. coli*, in relation to ZNOE, the inhibition zone noted were 21.33 ± 1.53 mm, ZNOA 18.67 ± 1.53 mm, and ZNOAE 20.33 ± 1.53 mm. However, zones of inhibition for *E. faecalis* by ZNOE were 14.33 ± 2.08 mm, ZNOA 18.67 ± 2.08 mm, and ZNOAE 24.33 ± 1.53 mm.

Table 3 represents intergroup MIC comparisons of all test materials against test bacteria. MIC values of group I against *E. coli* and *E. faecalis* were detected at 250 and 500 µg/mL, correspondingly. Group II showed MIC values of 250 µg/mL against both *E. coli* and

Table 4: Intragroup comparisons of MIC values of test materials against test bacteria

Bacteria	*Comparison*	*f*	*p*	*Inference*
E. Coli	Groups I and II	(not derived)	–	NS
	Groups I and III			NS
	Groups II and III			NS
E. faecalis	Groups I and II	(not derived)	–	NS
	Groups I and III			NS
	Groups II and III			NS

Where; *f*, statistic value; *p*, probability value; NS, nonsignificant ($p > 0.05$); S, significant ($p < 0.05$); HS, highly significant ($p < 0.001$)

E. faecalis, whereas group III demonstrated 125 and 250 µg/mL against *E. coli* and *E. faecalis*, respectively. No significant difference was observed among the test agents and bacteria for MIC.

Table 4 represents intragroup comparisons of MIC values of all the test materials against the test bacteria performed by unpaired *t*-test. Statistically, no significant difference was detected in intragroup comparisons for all the text agents against all the test bacteria for MIC. The *f*-statistics values for intragroup comparison were not derived because similar observation values created errors during calculation, hence the results.

Table 5 represents an intergroup comparison of the MBC of test materials. The mean difference in MBC values of group I was found to be 4.696. The p-value was 0.009, which was HS. There was a mean difference of −4.96 for the MBC values of group II, with the *p*-value of 0.009 representing a highly statistically significant difference. The mean difference in MBC values of group III was −3.207. The *p*-value was 0.033, indicating a statistically significant difference.

Table 6 represents an intragroup comparison of the MBC of test materials. The individual mean difference for pairwise comparison of groups was made against *E. coli* and *E. faecalis*. When group I was compared with group II, the mean difference in MBC values was found to be 2.13, while the *p*-value was 0.09. The mean difference between groups I and III was 0.8, with a *p*-value of 0.46. When group II was compared with group III, the mean difference in MBC values was found to be −1.33, while the *p*-value was 0.25. Thus, the individual pairwise comparison performed by unpaired *t*-test showed some difference between the three groups against *E. coli*, which was not statistically significant.

Table 5: Intergroup comparison of MBC of test materials

Groups	*Bacteria*	*N*	*Mean*	*SD*	*t*	*p*	*Inference*
I (ZNOE)	*E. coli*	3	21.33	1.53	4.696	0.009	HS
	E. faecalis	3	14.33	2.08		(<0.01)	
II (ZNOA)	*E. coli*	3	18.67	1.53	−4.696	0.009	HS
	E. faecalis	3	25.67	2.08		(<0.01)	
III (ZNOAE)	*E. coli*	3	20.33	1.53	−3.207	0.033	S
	E. faecalis	3	24.33	1.53		(<0.05)	

Where; SD, standard deviation; *N*, number; *t*, unpaired *t*-test; *p*, probability value; NS, nonsignificant ($p > 0.05$); S, significant ($p < 0.05$); HS, highly significant ($p < 0.001$)

Table 6: Intragroup comparison of MBC of all test materials against *E. coli* and *E. faecalis*

Bacteria	*Comparison*	*t*	*p*	*Inference*
E. coli	Groups I and II	2.13	0.09	NS
	Groups I and III	0.8	0.46	NS
	Groups II and III	−1.33	0.25	NS
E. faecalis	Groups I and II	−6.66	0.003	S
	Groups I and III	−6.7	0.003	S
	Groups II and III	0.89	0.42	NS

Where; *t*, unpaired *t*-test; *p*, probability value; NS, nonsignificant ($p > 0.05$); *S*, significant ($p < 0.05$); HS, highly significant ($p < 0.001$)

The individual mean difference for the pairwise comparison of groups was made against *E. faecalis*. When group I was compared with group II, the mean difference in MBC values was found to be −6.66, while the *p*-value was 0.003. The mean difference in groups I and III was −6.7, with a *p*-value of 0.003. When group II was compared with group III, the mean difference in MBC values was found to be 0.89, while the *p*-value was 0.42. Thus, the individual pairwise comparison shows some difference among the three groups against *E. faecalis*, which was statistically significant when performed by an unpaired *t*-test.

Discussion

Elimination of infection by chemomechanical and biomechanical preparation is the principal objective of endodontic therapy.[17] The complex anatomy of endodontic spaces makes cleaning and shaping difficult. Moreover, wide medullary bone spaces favor the fast spread of infection.[23,24] Hence, root canal filling materials with good antimicrobial properties have been proven effective in the endodontic therapy of primary teeth.[7]

Chisholm first used ZNOE in dentistry.[25] Being the most commonly used obturating material, the various disadvantages associated with its use in primary teeth led to the invention of numerous other materials like $Ca(OH)_2$, Kri paste, Endoflas, Vitapex, etc.[8] Various modifications have been done on ZNOE to improve its properties. Even essential oils are herbal phenolic compounds harboring bactericidal properties.[26] Ajwain oil has various medicinal properties and is used in multiple products.[27] Antibacterial efficacy of ajwain oil against different bacteria is mainly due to main ingredients such as thymol and carvacrol.[28]

E. coli are gram-negative, facultative anaerobes that are commensals in the lower intestinal tract. Pathogenic *E. coli* acquires multiple virulence determinants, such as toxins, adhesins, and proteins. They are mainly isolated from persistent periapical infections, and their endotoxin plays a crucial role in exacerbating the infectious stage.[8] The oral cavity and gingival sulcus harbor facultative anaerobic gram positive *E. faecalis*.[29] Various enzymes like lipoteichoic acid, cytolysin, and aggregation substances exert virulence factors of *E. faecalis,* making its persistent presence in failed root canal treatments.[30]

The current study utilized a mixture of zinc oxide with eugenol and ajwain oil and a combination of ajwain and eugenol, and their antibacterial efficiency was appraised and compared against *E. coli* and *E. faecalis*. ZNOE acts as a control group. The data obtained from the current study demonstrated ZNOE inhibiting *E. coli* at 250 µg/mL and *E. faecalis* at 500 µg/mL concentration, indicating its better efficacy against *E. coli*. MBC values determined against *E. coli* and *E. faecalis* were 21.33 and 14.33 mm, respectively, indicating medium inhibition of *E. faecalis* and potent inhibition against *E. coli*. The results obtained as inhibition and killing of bacteria by ZNOE were probably due to eugenol content. A study demonstrated no inhibitory effect of zinc oxide powder but with the addition of eugenol-enhanced inhibition of gram positive bacteria. Eugenol causes protein denaturation, leading to bacterial cell death, but eugenol is potentially cytotoxic, and its toxicity decreases after 1–7 days.[31] Various studies have evaluated the antibacterial activities of ZNOE,[1,17,32,33] and the outcomes of the current experiment were inclined to the effects mentioned by these studies.

Zinc oxide mixed with ajwain oil (ZNOA) showed inhibition of *E. coli* and *E. faecalis* at 250 µg/mL concentration, representing its better efficacy. MBC values determined against *E. coli* and *E. faecalis* were 18.67 and 25.67 mm, respectively, indicating intermediate inhibition of *E. coli*, besides stout inhibition of *E. faecalis*. The extracts of ajwain oil, like thymol, γ-terpinene, O-cymene, diallyl sulfur, carvacrol, phenol, allicin, and luteolin, possess potent antibacterial activity.[34] Besides, thymol eliminates multidrug-resistant and third-generation antibiotic-resistant bacteria. It works by damaging the bacterial cell membrane, altering its pH and physiologic equilibrium, and thus causing membrane rupture as well as loss of cellular contents. p-cymene, a hydrophobic molecule, acts as a phenolic compound enhancer along with thymol, augmenting cell membrane distension.[7,28] Secondary metabolites include essential oils, tannins, limonoids, glycosides, coumarins, alkaloid quinones, saponins, phenols, flavones, and flavonoids from the ajwain oil, inhibit cellular breathing, causing high membrane permeability and the death of bacteria.[35] The results of the current experiment align with the outcomes of Kaur and Arora and Shah et al. demonstrating better efficacy of ajwain oil against *E. faecalis*.[11,36]

Likewise, ZNOAE demonstrated better antibacterial activity against *E. coli* at 125 µg/mL and *E. faecalis* at 250 µg/mL. The inhibition zones against *E. coli* and *E. faecalis* were 20.33 and 24.33 mm, correspondingly indicating strong inhibition of both bacteria. A thorough examination of the literature search engines could not support any study or literature discussing the mechanism

of action of ZNOAE. This combination has been tried for the first time, with promising results comparable to ZNOE and ZNOA. The probable reason for the better results can be attributed to the synergetic effect of eugenol, thymol, and carvacrol in ajwain oil. Pei et al. demonstrated eugenol combined with carvacrol or thymol enhances bacterial cell membrane damage, allowing permeation of the eugenol into the cytoplasm and deactivating cell proteins.[37,38] Thymol or carvacrol also upsurges the cytoplasmatic membrane absorbency, hence showing the desirable effect.[39]

Thus, the new blends of zinc oxide with ajwain oil and eugenol showed better efficacy vs *E. faecalis* than ZNOE. They also showed comparable antibacterial efficiency against *E. coli* compared to ZNOE. Based on the results obtained for all test materials, the suggested combinations can be considered substitutes for ZNOE as primary teeth obturating materials. However, further clinical and *in vitro* studies will be required to determine other properties of these new combinations to be used as endodontic filling materials.

Limitations of the Study

Though the test materials have shown better antimicrobial effects than conventional ones, the study has certain limitations

- Being *in vitro* study, clinical conditions can't be replicated.
- The financial constraints limited the use of two bacterial strains.
- A comparison of newly formulated test materials was made with only one material.

Conclusion

Based on the employed methodology and according to the results obtained, it can be concluded that a newly formulated combination of materials can be considered a viable alternative to traditional material ZNOE in terms of better antimicrobial efficacy against resistant endodontic pathogens foreseeable endodontic results. Further clinical studies are recommended for forgeable and stouter speculations.

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