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Comparative Evaluation of Smear Layer Removal and Antibacterial Efficacy of Neem Extract and Neem–Chitosan Combination with Conventional Irrigation Protocol: An in Vitro Scanning Electron Microscopic Study

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Abstract: Successful Endodontic Treatment Depends On Effective Root Canal Disinfection And Smear Layer Removal. Conventional Irrigants Such As Sodium Hypochlorite And Edta Are Effective But Possess Certain Limitations Including Cytotoxicity And Dentinal Alterations. Herbal Irrigants And Biopolymers Have Gained Attention As Potential Biocompatible Alternatives.

To Comparatively Evaluate The Smear Layer Removal And Antibacterial Efficacy Of Neem Extract And Neem–Chitosan Combination With Conventional Irrigation Protocol Using Scanning Electron Microscopy (Sem) And Mic$_{50}$ Analysis.

Forty Extracted Human Single-Rooted Teeth Were Decoronated And Biomechanically Prepared. Samples Were Randomly Divided Into Four Groups (N=10): Group I – Naocl + Edta, Group Ii – Neem Extract, Group Iii – Neem–Chitosan Combination, And Group Iv – Saline. Irrigation Protocols Were Ultrasonically Activated. Smear Layer Removal At The Apical Third Was Evaluated Using Sem And Scored According To Hülsmann's Criteria. Antibacterial Efficacy Was Assessed Using Mic$_{50}$ Microdilution Method Against *Escherichia Coli* And *Staphylococcus Aureus*. Statistical Analysis Was Performed Using Kruskal–Wallis And Dunn's Post Hoc Tests.

Group Iii Demonstrated The Lowest Mean Smear Layer Score (1.90 ± 0.74), Indicating Superior Smear Layer Removal, Followed By Group I (2.30 ± 0.67), Group Ii (3.10 ± 0.74), And Group Iv (4.40 ± 0.70). Intergroup Comparison Showed Statistically Significant Differences ($P < 0.001$). Mic$_{50}$ Analysis Demonstrated Highest Antibacterial Efficacy For Sodium Hypochlorite, Followed By Neem Extract, While Neem–Chitosan Showed Comparatively Reduced Antimicrobial Activity.

Neem–Chitosan Combination Demonstrated Superior Smear Layer Removal Under Sem Evaluation, Whereas Sodium Hypochlorite Exhibited Superior Antibacterial Efficacy. The Findings Suggest That Enhanced Smear Layer Removal Does Not Necessarily Correlate With Greater Antimicrobial Activity. A Multimodal Irrigation Approach Combining Effective Cleaning And Antimicrobial Action May Improve Endodontic Outcomes.

Keywords: *Neem Extract; Chitosan; Smear Layer; Sem; Root Canal Irrigants; Antibacterial Efficacy; Mic$_{50}$; Ultrasonic Activation.*

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I. INTRODUCTION

Successful endodontic therapy primarily depends on effective cleaning, shaping, and disinfection of the root canal system. Mechanical instrumentation alone is insufficient to completely eliminate microorganisms and debris from the complex anatomy of root canals, especially in fins, isthmuses, lateral canals, and dentinal tubules. Therefore, chemical irrigation plays a critical role in achieving adequate canal debridement and microbial control.[1,2]

During biomechanical preparation, instrumentation of dentin walls produces an amorphous layer known as the smear layer, which consists of organic and inorganic debris, remnants of odontoblastic processes, microorganisms, and necrotic tissue.[3] The smear layer can obstruct dentinal tubules, reduce the penetration of irrigants and intracanal medicaments, and compromise the adaptation and sealing ability of obturating materials.[4] Consequently, effective smear layer removal has become an important objective of contemporary endodontic irrigation protocols.

Sodium hypochlorite (NaOCl) remains the most commonly used endodontic irrigant because of its broad-spectrum antimicrobial activity and tissue-dissolving capability.[5] However, NaOCl alone is ineffective in removing the inorganic component of the smear layer and may cause cytotoxic reactions when extruded beyond the apex.[6] Ethylenediaminetetraacetic acid (EDTA) is commonly used as a chelating agent for smear layer removal, particularly the inorganic component.[7] Despite their effectiveness, conventional irrigants possess several disadvantages including dentinal erosion, unpleasant taste, toxicity, and alteration of dentin structure.[8] These limitations have encouraged researchers to explore herbal and biopolymer-based alternatives with improved biocompatibility.

Neem (*Azadirachta indica*) is a medicinal plant widely recognized for its antimicrobial, anti-inflammatory, antioxidant, and antifungal properties.[9] The antibacterial activity of Neem is attributed to bioactive constituents such as nimbidin, azadirachtin, flavonoids, tannins, and quercetin.[10] Previous studies have demonstrated that Neem extract exhibits inhibitory action against endodontic pathogens including *Enterococcus faecalis*, suggesting its potential use as a root canal irrigant.[11] In addition, herbal irrigants are generally considered more biocompatible and environmentally friendly compared to conventional chemical irrigants.

Chitosan is a natural polysaccharide derived from chitin and has gained attention in dentistry because of its biocompatibility, biodegradability, chelating ability, and antimicrobial properties.[12] Chitosan can interact with dentin through ionic interactions and has demonstrated the ability to remove the smear layer with minimal dentinal erosion.[13] Furthermore, nanoparticle and biopolymer forms of chitosan have shown promising antibacterial effects and enhanced penetration into dentinal tubules.[14]

Recently, interest has increased in combining herbal agents with biopolymers to enhance the overall efficacy of irrigating solutions. The combination of Neem extract and Chitosan may provide synergistic benefits by integrating the antimicrobial properties of Neem with the chelating and smear layer removal capabilities of Chitosan. However, limited literature is available evaluating the combined effect of Neem–Chitosan irrigant on both smear layer removal and antibacterial activity.

In addition to irrigant selection, activation methods significantly influence irrigation efficacy. Passive ultrasonic irrigation enhances irrigant penetration through acoustic streaming and cavitation, thereby improving debris removal and smear layer elimination, especially in the apical third.[15] Ultrasonic activation has also been reported to improve the antibacterial effectiveness of irrigating solutions by increasing their contact with canal walls and dentinal tubules.

Therefore, the present in vitro study was undertaken to comparatively evaluate the smear layer removal efficacy and antibacterial activity of Neem extract and Neem–Chitosan combination with conventional irrigation protocol using scanning electron microscopy (SEM) and MIC_{50} analysis.

II. MATERIALS AND METHODS

This in vitro study was conducted in the Department of Conservative Dentistry and Endodontics, Jaipur Dental College and Hospital, Jaipur, in collaboration with the Department of Veterinary Microbiology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences (RAJUVAS), Bikaner, where scanning electron microscopic evaluation and microbiological analysis were performed.

➢ *Sample Selection*

Forty freshly extracted human single-rooted teeth with mature apices and straight canals were selected for the study. Teeth with root caries, cracks, fractures, calcified canals, internal or external resorption, and previously treated canals were excluded.

The collected teeth were cleaned of soft tissue remnants and stored in normal saline until use.

➢ *Sample Preparation*

Decoronation of all teeth was performed using a diamond disc under water coolant to standardize root length

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at 13 mm. Working length was established using a size #10 K-file and confirmed 1 mm short of the apical foramen.

Biomechanical preparation was performed using rotary instrumentation up to standardized apical enlargement. During instrumentation, canals were irrigated according to the respective experimental groups.

The samples were randomly divided into four groups (n = 10):

- ✓ *Group I:*
  Conventional irrigation protocol (2.5% sodium hypochlorite + 17% EDTA)

- ✓ *Group II:*
  5% Neem extract

- ✓ *Group III:*
  Neem–Chitosan combination

- ✓ *Group IV:*
  Normal saline (negative control)

➢ *Preparation of Experimental Irrigants*

- *Neem Extract*
  Fresh Neem leaves were processed to obtain aqueous Neem extract, which was filtered and prepared at standardized concentration.

- *Chitosan Solution*
  A 0.2% Chitosan solution was prepared using appropriate solvent under magnetic stirring until complete dissolution was achieved.

- *Neem–Chitosan Combination*
  Equal volumes of Neem extract and Chitosan solution were mixed using a vortex mixer to obtain a homogenous experimental irrigant.

Irrigation was performed using side-vented irrigation needles. Ultrasonic activation of the irrigants was carried out in all experimental groups to enhance irrigant penetration and smear layer removal.

Following completion of irrigation protocols, canals were flushed with distilled water and dried using absorbent paper points.

➢ *SEM Evaluation*

The roots were longitudinally sectioned to obtain two halves. Specimens were dehydrated in ascending concentrations of ethanol and subjected to critical point drying. The samples were then mounted on aluminum stubs and gold sputter-coated to improve electrical conductivity during SEM imaging.

Scanning electron microscopic evaluation was performed at the apical third under different magnifications. Smear layer removal was assessed according to Hülsmann's 5-point scoring criteria:

- *Score 1:*
  No smear layer; all dentinal tubules open.

- *Score 2:*
  Small amount of smear layer; some tubules open.

- *Score 3:*
  Homogenous smear layer covering the surface; few tubules open.

- *Score 4:*
  Complete surface covered by smear layer; no open tubules.

- *Score 5:*
  Heavy non-homogenous smear layer covering the entire surface.

➢ *Antibacterial Evaluation*

Antibacterial efficacy was evaluated using the microdilution MIC_{50} method against *Escherichia coli* and *Staphylococcus aureus.*

Serial dilutions of the experimental irrigants were prepared in microtiter plates, followed by inoculation with bacterial suspensions. The plates were incubated at 37°C, after which resazurin dye was added as an indicator of bacterial viability.

Optical density readings at 600 nm were obtained using a SpectraMax iD5 microplate reader. MIC_{50} values were calculated using the Reed–Muench method.

➢ *Statistical Analysis*

Data were entered into Microsoft Excel and analyzed using SPSS software version 20. Descriptive statistics including mean and standard deviation were calculated.

Intergroup comparison of smear layer scores was performed using the Kruskal–Wallis test, followed by Dunn's post hoc analysis for pairwise comparison.

A p-value of less than 0.05 was considered statistically significant.

III. RESULTS

➢ *Smear Layer Removal Evaluation (SEM Analysis)*

Smear layer removal was evaluated under scanning electron microscopy (SEM) at the apical third using Hülsmann's 5-point scoring system. Lower scores indicated better smear layer removal and greater dentinal tubule patency, whereas higher scores indicated increased smear layer persistence and dentinal tubule occlusion.

➢ *Group I (NaOCl + EDTA)*

SEM analysis of Group I specimens revealed relatively clean dentinal surfaces with partially to completely open dentinal tubules. Most samples demonstrated a thin smear layer with mild remnants, particularly in the apical region. Occasional areas showed moderate smear layer persistence, which could be attributed to anatomical complexity and limited irrigant penetration in the apical third.

The mean smear layer score for Group I was 2.30 ± 0.67, indicating good smear layer removal.

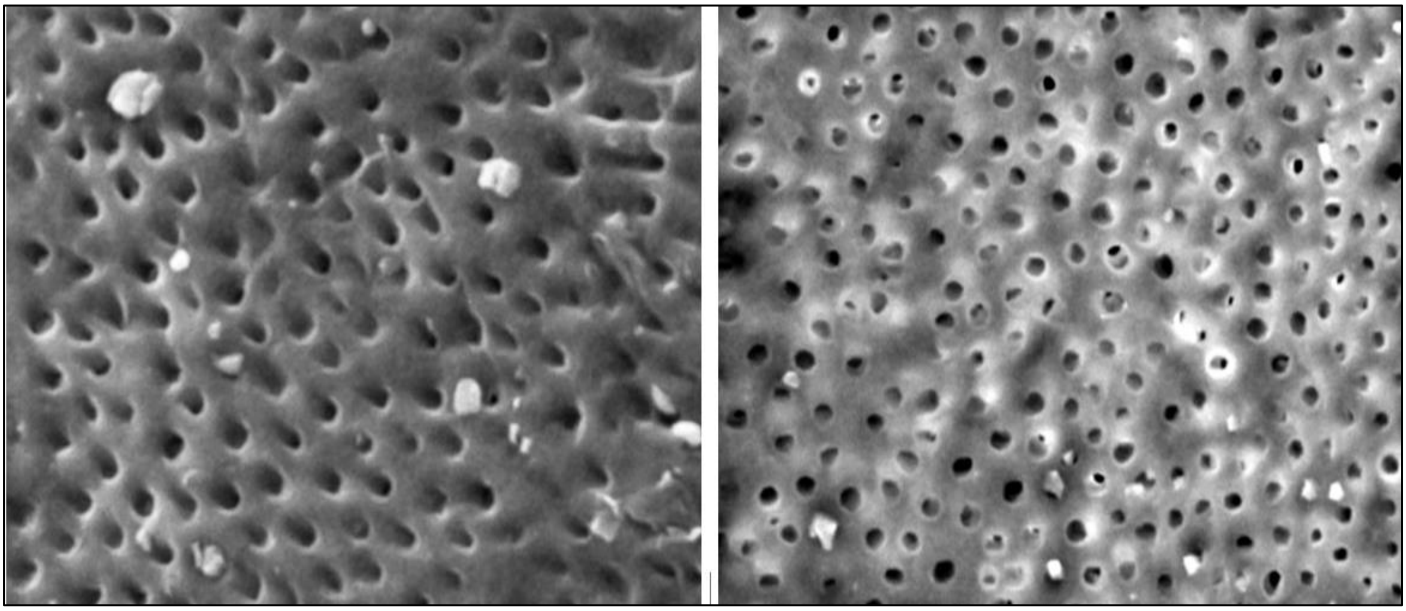

Fig 1 SEM Images of NaOCl and EDTA Group

➢ *Group II (Neem Extract)*

SEM evaluation of Group II showed moderate smear layer removal. Dentinal tubules were partially visible; however, smear layer remnants and debris were evident in several areas. The surface appeared heterogeneous, with incomplete cleaning and occasional occlusion of dentinal tubules.

The mean smear layer score for Group II was 3.10 ± 0.74, indicating moderate efficacy.

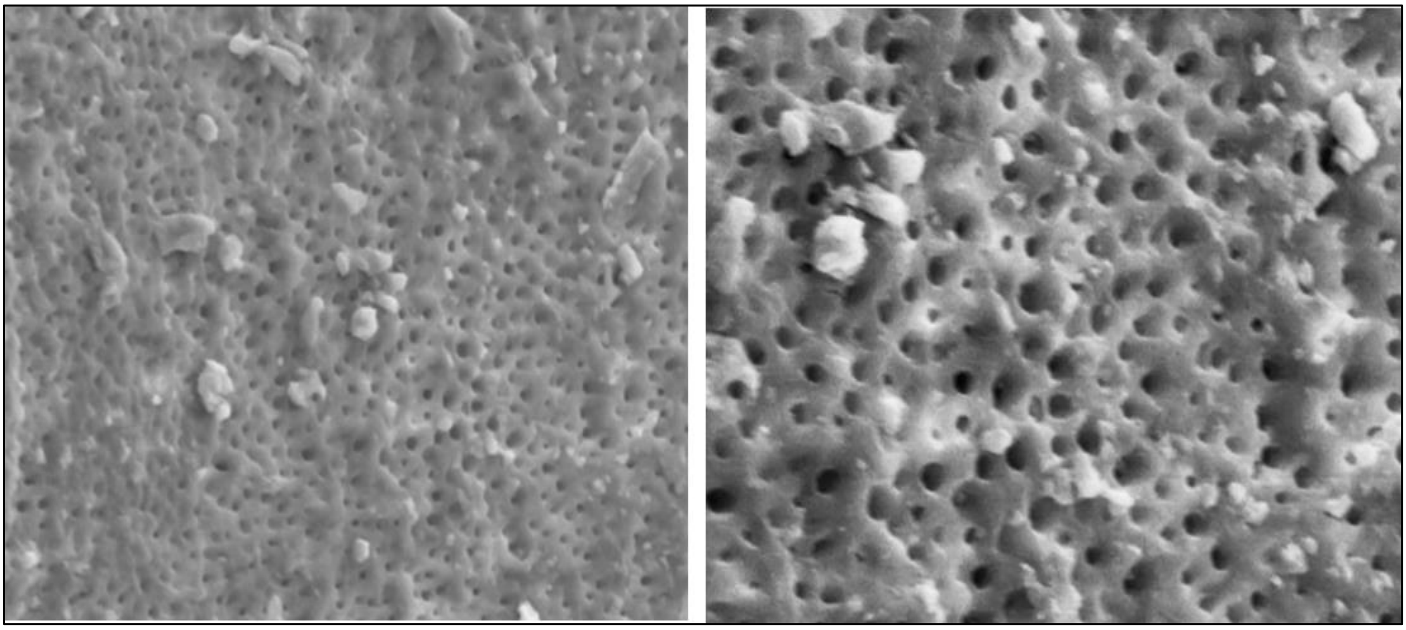

Fig 2 SEM Images of Neem Group

➢ *Group III (Neem–Chitosan Combination)*

SEM images of Group III demonstrated the most effective smear layer removal. The dentinal surfaces appeared cleaner with a large number of open dentinal tubules and minimal smear layer remnants. The cleaning pattern was consistent across samples, with only occasional areas showing mild smear persistence.

The mean smear layer score for Group III was 1.90 ± 0.74, indicating superior smear layer removal efficacy.

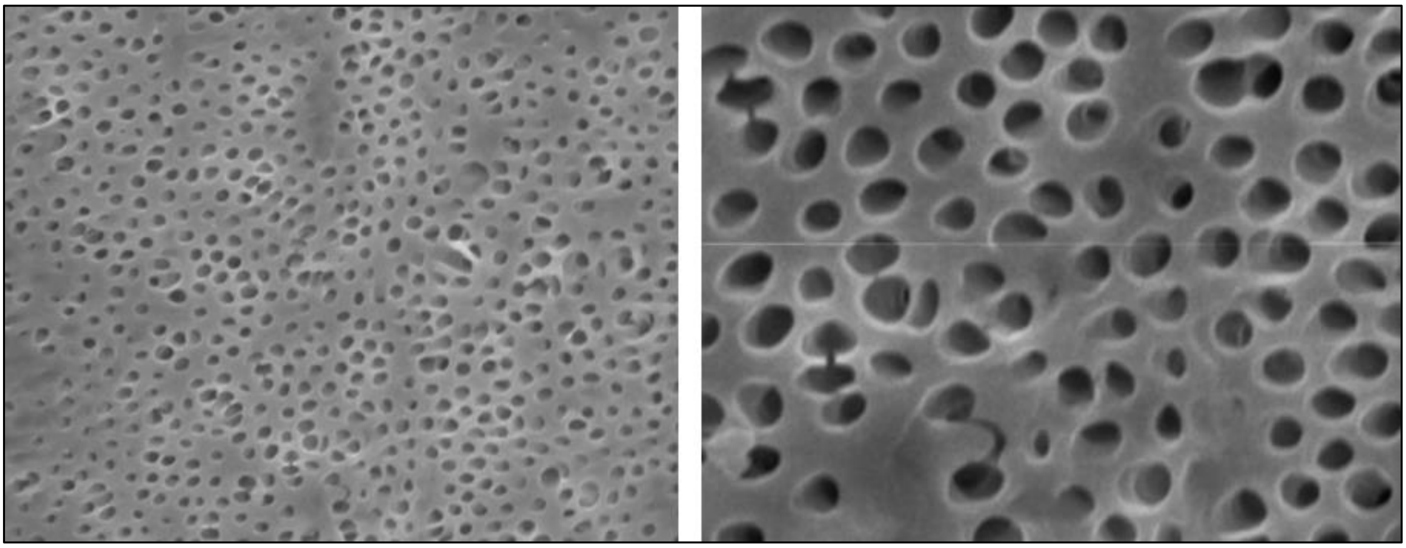

Fig 3 SEM Images of Neem - Chitosan Combination

➢ *Group IV (Saline – Negative Control)*

SEM analysis of Group IV revealed heavy smear layer coverage with complete occlusion of dentinal tubules. The dentin surface appeared irregular with debris accumulation and absence of clean tubule openings, confirming the ineffectiveness of saline in smear layer removal.

The mean smear layer score for Group IV was 4.40 ± 0.70, indicating poor smear layer removal.

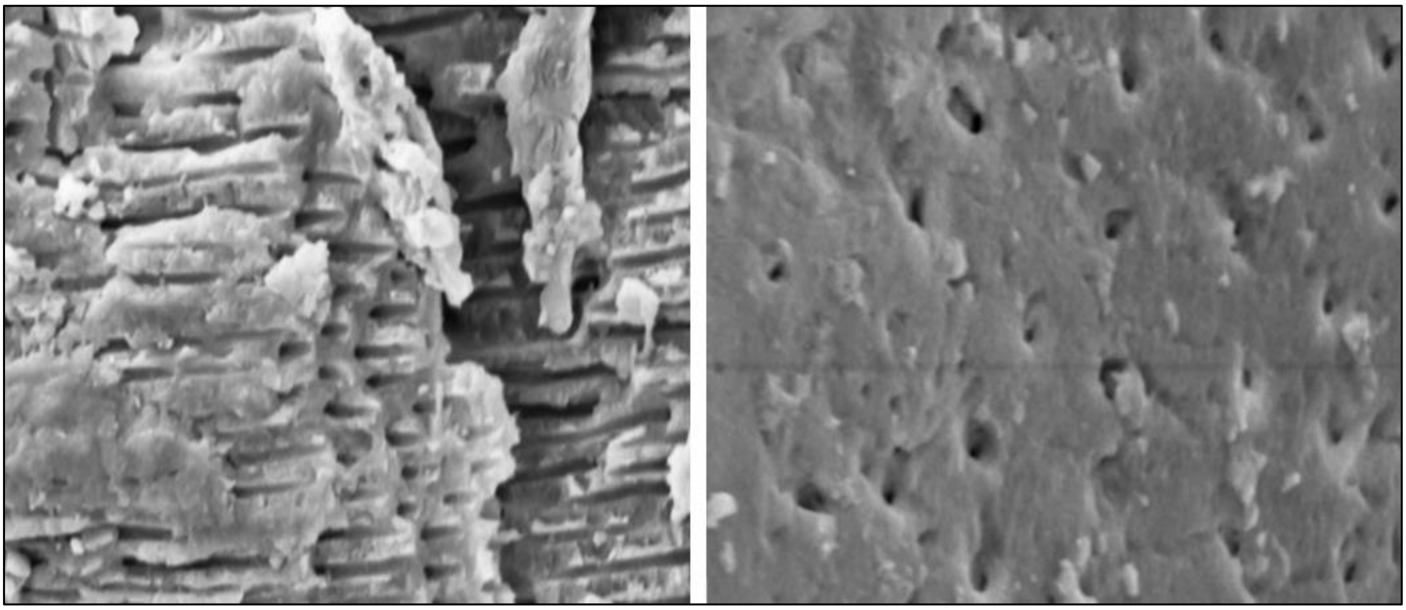

Fig 4 SEM images of Saline group

➢ *Distribution of Smear Layer Scores*

Table 1 Distribution of Hülsmann Scores Among the Study Groups

Hülsmann Score	Group I NaOCl + EDTA	Group II Neem	Group III Neem–Chitosan	Group IV Saline
Score 1	1 (10%)	0 (0%)	3 (30%)	0 (0%)
Score 2	5 (50%)	2 (20%)	5 (50%)	0 (0%)
Score 3	4 (40%)	5 (50%)	2 (20%)	1 (10%)
Score 4	0 (0%)	3 (30%)	0 (0%)	4 (40%)
Score 5	0 (0%)	0 (0%)	0 (0%)	5 (50%)
Total	10	10	10	10

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Majority of specimens in Group III exhibited scores 1 and 2, whereas Group IV predominantly demonstrated scores 4 and 5.

- *Descriptive Statistics*

Table 2 Descriptive Statistics of Smear Layer Scores

Group	Mean ± SD	Median	Interpretation
Group III	1.90 ± 0.74	2	Best smear layer removal
Group I	2.30 ± 0.67	2	Good smear layer removal
Group II	3.10 ± 0.74	3	Moderate smear layer removal
Group IV	4.40 ± 0.70	4.5	Poor smear layer removal

Order of Smear Layer Removal Efficacy:

Group III > Group I > Group II > Group IV

- *Intergroup Comparison*

Table 3 Kruskal–Wallis Test

Test	H-value	p-value	Significance
Kruskal–Wallis	25.39	<0.001	Highly significant

A Statistically Highly Significant Difference was Observed among the Groups.

- *Pairwise Comparison (Dunn's Post Hoc Analysis)*

Table 4 Intergroup Comparison

Comparison	Interpretation	Significance
Group III vs Group I	Slightly better performance	Not significant
Group III vs Group II	Better than Neem	Significant
Group III vs Group IV	Much better than saline	Highly significant
Group I vs Group II	Better than Neem	Not significant
Group I vs Group IV	Better than saline	Highly significant
Group II vs Group IV	Better than saline	Significant

- *Graphical Representation*

Graphical representation of the data was performed using:

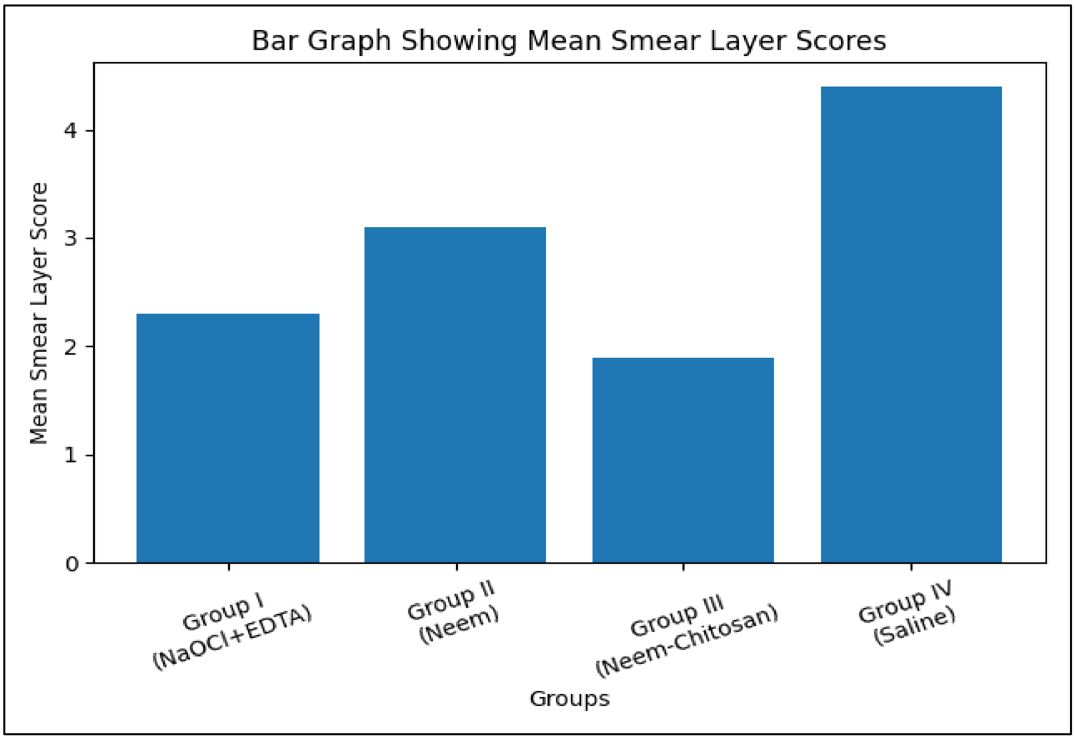

Fig 1 Bar Graph Showing Mean Smear Layer Scores Among Groups

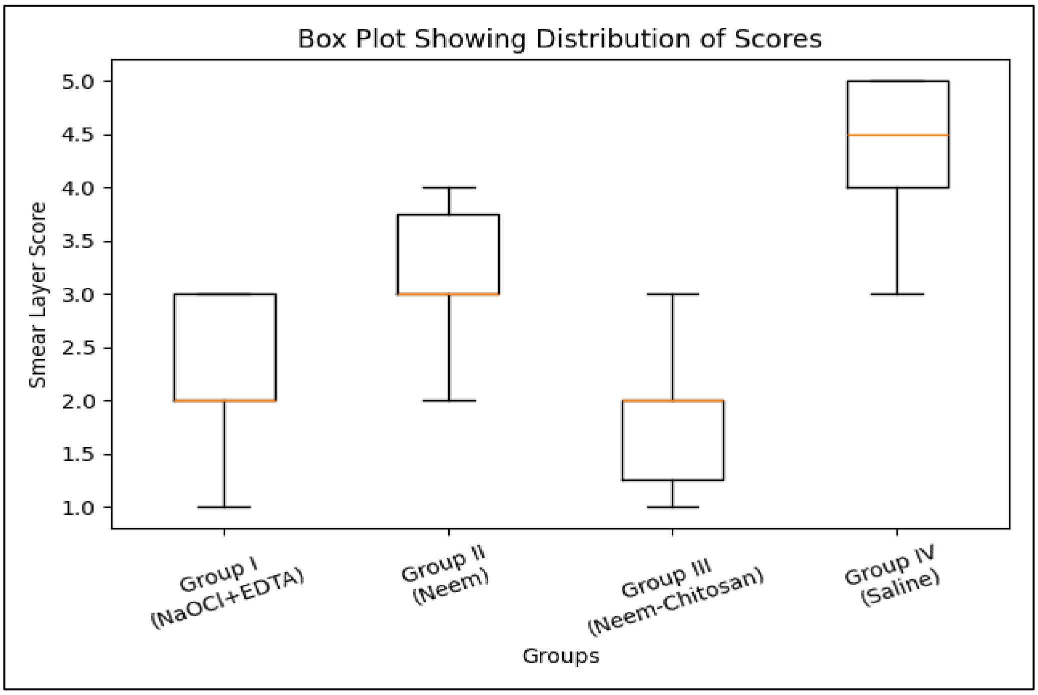

Fig 2 Box Plot Showing Distribution of Scores Among Groups

Lower mean scores indicated better smear layer removal.

IV. ANTIBACTERIAL EFFICACY (MIC_{50} ANALYSIS)

Antibacterial efficacy was evaluated using the microdilution method against *Escherichia coli* and *Staphylococcus aureus*. Optical density readings at 600 nm were recorded, and MIC_{50} values were calculated using the Reed–Muench method.

Table 5 MIC_{50} Values of Test Irrigants

Irrigant	**MIC_{50} (E. coli)**	**MIC_{50} (S. aureus)**	**Interpretation**
NaOCl	Lowest	Lowest	Highest antibacterial activity
Neem Extract	Moderate	Moderate	Moderate antibacterial activity
Neem–Chitosan	Higher MIC_{50}	Higher MIC_{50}	Reduced antibacterial activity
Saline	Not applicable	Not applicable	Negligible antibacterial activity

- *Interpretation of Antibacterial Results*

Sodium hypochlorite demonstrated the highest antibacterial efficacy, showing the lowest MIC_{50} values. Neem extract exhibited moderate antimicrobial activity, whereas the Neem–Chitosan combination showed comparatively reduced antibacterial efficacy. Saline demonstrated negligible antibacterial activity and served as the negative control.

- *Correlation Between SEM and Antibacterial Findings*

Although the Neem–Chitosan combination demonstrated superior smear layer removal under SEM evaluation, its antibacterial efficacy was lower than that of sodium hypochlorite and Neem extract. This indicates that enhanced smear layer removal does not necessarily correlate with superior antimicrobial activity.

SEM analysis demonstrated that the Neem–Chitosan combination exhibited the best smear layer removal, followed by NaOCl + EDTA, Neem extract, and saline.

MIC_{50} analysis revealed that sodium hypochlorite exhibited the highest antibacterial efficacy, followed by Neem extract, whereas the Neem–Chitosan combination demonstrated comparatively reduced antimicrobial activity.

V. DISCUSSION

Successful endodontic therapy depends on effective cleaning and disinfection of the root canal system. Mechanical instrumentation alone is insufficient to eliminate microorganisms and debris from the intricate root canal anatomy; therefore, chemical irrigation plays a crucial role in achieving optimal canal debridement and microbial reduction. The present in vitro study comparatively evaluated the smear layer removal and antibacterial efficacy of Neem extract and Neem–Chitosan combination with conventional irrigation protocol using SEM analysis and MIC_{50} evaluation.

The smear layer formed during instrumentation consists of organic and inorganic debris that may harbor microorganisms and obstruct dentinal tubules, thereby limiting the penetration of irrigants, intracanal medicaments,

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and sealers. Hence, effective smear layer removal is considered essential for improved disinfection and successful obturation.[1,3]

In the present study, the Neem–Chitosan combination demonstrated the lowest mean smear layer score and the best overall cleaning efficacy under SEM evaluation. SEM images revealed cleaner dentinal surfaces with greater dentinal tubule patency and minimal smear layer remnants in Group III specimens. The enhanced smear layer removal observed with the Neem–Chitosan combination may be attributed primarily to the chelating property of Chitosan. Chitosan is a natural polysaccharide capable of interacting with dentin through ionic exchange, thereby facilitating removal of the inorganic component of smear layer.[28,30,31]

Previous studies have similarly reported the smear layer removing ability of Chitosan-based irrigants. Abdelkafy et al.[30] demonstrated that Chitosan nanoparticles effectively removed smear layer while preserving dentinal mineral content. Al-Hashimy and Al-Jadwaa[31] also observed superior dentinal tubule cleanliness with nano-Chitosan solutions. Ferreira-Reguera et al.[34], in a systematic review, concluded that Chitosan exhibits significant smear layer removal efficacy with less dentinal erosion compared to conventional chelators such as EDTA.

The superior cleaning efficacy observed in the present study may also be influenced by ultrasonic activation. Passive ultrasonic irrigation improves irrigant penetration through acoustic streaming and cavitation, thereby increasing smear layer disruption and debris removal, particularly in the apical third.[8,9,38] Several authors have emphasized the importance of activation techniques in enhancing irrigant effectiveness. Krishnegowda et al.[26] and Chavate et al.[25] demonstrated significantly improved smear layer removal with ultrasonic activation compared to conventional irrigation methods.

Group I (NaOCl + EDTA) also demonstrated effective smear layer removal, although slightly inferior to the Neem–Chitosan combination. This finding is consistent with previous literature reporting that EDTA effectively removes the inorganic component of smear layer, while sodium hypochlorite contributes organic tissue dissolution and antimicrobial action.[7,52] Baumgartner and Mader[7] demonstrated that combined NaOCl and EDTA irrigation protocols remain highly effective for canal cleanliness.

Group II (Neem extract) showed moderate smear layer removal. Although dentinal tubules were partially visible, smear layer remnants and debris persisted in several specimens. The comparatively lower efficacy may be related to the absence of strong chelating action within Neem extract alone. Nevertheless, the herbal solution demonstrated better smear layer removal than saline, indicating some cleaning potential.

Group IV (saline) demonstrated the poorest smear layer removal, with heavy smear layer deposition and complete occlusion of dentinal tubules. Saline lacks both chelating and tissue-dissolving properties and therefore served as an effective negative control in the present study.

The antibacterial evaluation in the present study demonstrated that sodium hypochlorite exhibited the highest antimicrobial efficacy, followed by Neem extract, whereas the Neem–Chitosan combination showed comparatively reduced antibacterial activity. Sodium hypochlorite remains the gold standard irrigant because of its potent broad-spectrum antimicrobial action and ability to dissolve necrotic tissue.[5,53]

Neem extract demonstrated moderate antibacterial efficacy against *Escherichia coli* and *Staphylococcus aureus*. The antimicrobial activity of Neem is attributed to phytochemical constituents such as nimbidin, azadirachtin, flavonoids, tannins, and quercetin.[18] Previous studies have similarly demonstrated the antibacterial potential of Neem against endodontic pathogens. Dutta and Kundabala[19] and Ghonmode et al.[20] reported significant antimicrobial activity of Neem extract against *Enterococcus faecalis*. Babaji et al.[21] also demonstrated appreciable antibacterial efficacy of Neem compared with other herbal irrigants.

Interestingly, despite superior smear layer removal, the Neem–Chitosan combination demonstrated comparatively lower antibacterial activity than Neem extract alone. This finding suggests that enhanced smear layer removal does not necessarily correlate with superior antimicrobial efficacy. One possible explanation may be the interaction between Neem phytochemicals and Chitosan molecules, which could alter the availability or diffusion of active antimicrobial constituents. The combined formulation may have enhanced dentin surface interaction and cleaning ability while simultaneously reducing release of active antibacterial components.

Another possible explanation may involve the physicochemical behavior of Chitosan within the irrigant solution. Chitosan exhibits strong bioadhesive properties and interacts readily with dentin surfaces, potentially enhancing smear layer removal but limiting antimicrobial diffusion within the broth medium used for MIC_{50} analysis. Similar observations have been discussed in studies evaluating nanoparticle-based and biopolymer irrigants.[28,32]

The present study also reinforces the concept that no single irrigant fulfills all the requirements of an ideal root canal irrigant. While Chitosan-containing formulations may provide superior smear layer removal and biocompatibility, sodium hypochlorite continues to demonstrate unmatched antimicrobial effectiveness. Therefore, combined irrigation protocols and activation techniques may offer superior clinical outcomes by integrating the advantages of different irrigating solutions.

The statistical analysis demonstrated a highly significant difference among the groups ($p < 0.001$), indicating that the observed differences in smear layer removal were not due to chance. Group III showed statistically superior cleaning compared to Neem and saline

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groups, supporting the effectiveness of Chitosan-containing formulations in smear layer management.

The findings of the present study possess important clinical implications. Effective smear layer removal enhances dentinal tubule penetration of irrigants, medicaments, and sealers, potentially improving the long-term success of endodontic therapy. Herbal and biopolymer-based irrigants may provide safer and more biocompatible alternatives for patients sensitive to conventional chemical agents. However, their antimicrobial limitations suggest that they should currently be considered adjuncts rather than complete replacements for sodium hypochlorite.

The present study has certain limitations. Being an in vitro study, it could not completely reproduce the complex clinical environment of the root canal system. Only selected bacterial strains were evaluated, and long-term substantivity and biocompatibility of the experimental irrigants were not assessed. Further in vivo and clinical studies with larger sample sizes are necessary to validate these findings and evaluate the long-term effectiveness of Neem–Chitosan formulations in endodontic therapy.

VI. CONCLUSION

Within the limitations of the present in vitro study, it can be concluded that the Neem–Chitosan combination demonstrated superior smear layer removal efficacy under scanning electron microscopic evaluation, particularly in the apical third, followed by the conventional NaOCl + EDTA protocol, Neem extract, and saline.

Sodium hypochlorite exhibited the highest antibacterial efficacy during MIC_{50} evaluation, whereas Neem extract demonstrated moderate antimicrobial activity. Although the Neem–Chitosan combination showed enhanced smear layer removal, its antibacterial efficacy was comparatively reduced, indicating that improved canal cleanliness does not necessarily correlate with superior antimicrobial action.

The findings of the present study suggest that Chitosan-containing irrigants possess promising chelating and smear layer removal properties and may serve as useful adjuncts in endodontic irrigation. However, conventional irrigants such as sodium hypochlorite continue to remain superior in terms of antimicrobial effectiveness.

Therefore, a multimodal irrigation strategy combining effective smear layer removal, antimicrobial activity, and activation techniques such as ultrasonics may provide improved clinical outcomes in endodontic therapy.

Further in vivo and long-term clinical studies are recommended to evaluate the substantivity, biocompatibility, and clinical applicability of Neem–Chitosan irrigating formulations.

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