

Comparative Evaluation of Ecoenzyme and EDTA for Smear Layer Removal and Their Effects on Root Dentin Microhardness: *An In Vitro* Study

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ABSTRACT:

The removal of the smear layer following root canal instrumentation is a critical step in endodontic therapy, as its persistence can compromise sealer penetration, dentinal tubule adaptation, and the overall success of obturation. Ethylenediaminetetraacetic acid (EDTA) has long been regarded as the gold-standard chelating agent for smear layer removal; however, concerns regarding its cytotoxicity, cost, and environmental impact have prompted interest in biocompatible, eco-friendly alternatives such as eco-enzyme, a fermented organic solution derived from fruit/vegetable waste, jaggery, and water.

Aim: To comparatively evaluate the smear layer removal efficacy of eco-enzyme and EDTA in extracted human teeth using scanning electron microscopy (SEM), an in vitro study.

Materials and Methods: Forty eight extracted single-rooted human permanent teeth were selected, decoronated, and biomechanically prepared. Samples were randomly divided into groups: Group I – EDTA (17%) final rinse, Group II – Eco-enzyme final rinse, and Group III – control/saline. Teeth were sectioned longitudinally and examined under SEM at the coronal, middle, and apical thirds. Smear layer removal was scored using Hülsmann's criteria. Data were statistically analyzed using

Results: Both eco-enzyme and EDTA significantly outperformed normal saline at all three levels of root canal (all adjusted $p < 0.02$). Vickers microhardness analysis showed a corresponding, expected reduction in dentin microhardness following irrigation, with EDTA producing the greatest reduction, followed by eco-enzyme, and saline producing minimal change.

Conclusion: Eco-enzyme demonstrated smear layer removal efficacy statistically comparable to 17% EDTA at the coronal, middle, and apical thirds, while causing less reduction in dentin microhardness. These findings support eco-enzyme's potential as a biocompatible, eco-friendly alternative irrigant in endodontic practice.

Keywords: Smear layer, EDTA, eco-enzyme, root canal irrigation, scanning electron microscopy, chelating

agent.

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INTRODUCTION:

The primary objective of root canal therapy is the thorough elimination of microorganisms, necrotic debris, and pulpal remnants from the root canal system, followed by three-dimensional obturation to achieve a hermetic seal.¹ During instrumentation, mechanical action of endodontic files against the dentinal walls produces an irregular, amorphous layer of debris known as the smear layer.² This layer consists of an organic component — remnants of pulp tissue, odontoblastic processes, and microorganisms and an inorganic component comprising calcified dentinal particles, which becomes packed into the dentinal tubules as a plug of varying thickness.^{2,3}

The clinical significance of the smear layer has been a subject of extensive debate. While it may occlude dentinal tubules and theoretically reduce bacterial penetration, most contemporary evidence suggests that its presence is detrimental, as it harbours bacteria and their by-products, interferes with the intimate adaptation of root canal sealers and obturating materials to the dentinal walls, and compromises the integrity of the apical seal.^{3,4}

Consequently, its removal prior to obturation is widely advocated to enhance the penetration of irrigants and sealers into dentinal tubules, improve bond strength, and reduce microleakage.^{4,5}

A variety of chelating and demineralizing agents have been employed to remove the smear layer, of which 17% EDTA (Ethylenediaminetetraacetic acid) remains the most extensively studied and widely used.⁶

EDTA acts by chelating calcium ions from the dentin surface, thereby dissolving the inorganic component of the smear layer and opening dentinal

tubules.^{6,7}

Despite its efficacy, EDTA is not without limitations — prolonged or repeated exposure has been associated with excessive peritubular and intertubular dentin erosion, cytotoxic effects on periapical tissues, and reduced sealer bond strength when overused.^{8,9} Additionally, the synthetic nature and disposal of EDTA raise concerns regarding biocompatibility and environmental sustainability.

These limitations have driven interest toward natural, biocompatible alternatives. Eco-enzyme, also referred to as "garbage enzyme," is a fermented solution prepared from fruit or vegetable peels, jaggery (or sugar), and water over a period of several weeks.^{10,11}

This fermentation process yields a complex mixture rich in organic acids — including acetic acid, citric acid, and other weak acids — along with enzymes possessing proteolytic, lipolytic, and antimicrobial properties.

Owing to its acidic pH and chelating potential, eco-enzyme has been proposed as an economical, eco-friendly, and biocompatible irrigant with possible applications in smear layer removal; recent work has demonstrated antimicrobial efficacy of fruit-peel eco-enzymes against *Enterococcus faecalis* comparable to 2.5% sodium hypochlorite, though its efficacy relative to conventional chelating agents for smear layer removal remains poorly characterized in the endodontic literature.¹⁰

Given the paucity of comparative data evaluating eco-enzyme against the established efficacy of EDTA, there exists a clear need for controlled in vitro investigation. This study was therefore undertaken to comparatively evaluate the smear layer removal efficacy of eco-enzyme and EDTA

RESEARCH PAPER

using scanning electron microscopic analysis, with the aim of exploring eco-enzyme's viability as a sustainable adjunct or alternative to conventional chelating irrigants in endodontic practice

Materials And Methodology:

The study was an experimental in vitro investigation that was created using the modified Consolidated Standards of Reporting Trials.

In the present study, forty eight mandibular premolars were used. G* power software (version 3.1.9.2) (Erdfelder, Faul, & Buchner, 1996) was used for determining the sample size, with 0.08 power, effect size = 0.371, significance level as = 0.05 and alpha error of 0.05.

The study was performed using forty eight extracted human mandibular premolar teeth. The selection criteria were teeth with relatively straight roots and mature root apex having patent canals without any anatomic variations were included. Teeth with calcified canals, fillings or posts, root resorption or perforations, caries, or fracture were excluded.

A total of **48 extracted single-rooted teeth** were used and divided into two main experimental arms:

Group A – Smear Layer Removal (n = 24)

- Group 1: Eco Enzyme
- Group 2: 17% EDTA
- Group 3: Normal saline

Group B – Root Dentin Microhardness (n = 24)

- Group 1: Eco Enzyme
- Group 2: 17% EDTA
- Group 3: Normal saline

Preparation of Eco-Enzyme:

Eco-enzyme was freshly prepared in the laboratory prior to use. Orange peel, lemon peel, jaggery, and water were combined in a ratio of 3:1:10 (citrus peels : jaggery : water) in an airtight fermentation container. The mixture was allowed to ferment at room temperature for a period of 3 months, with periodic release of gas to prevent container rupture during the fermentation process. Following the

completion of fermentation, the resultant solution was filtered through a muslin cloth to separate it from solid residues. The filtrate was then centrifuged at 10,000 rpm for 6 minutes to remove any remaining particulate matter, and the supernatant was filter-sterilized using a 0.45 µm sterile disposable syringe filter (Sartorius) to obtain a clear, microbiologically sterile eco-enzyme solution ready for experimental use. The prepared eco-enzyme, characteristic amber/orange in colour, was stored in an airtight sterile container at room temperature until use.



Fig 1: Ecoenzyme

Preparation Of Samples:

Each selected tooth was decoronated at the cemento-enamel junction using a diamond disc under copious water coolant to standardize root length across all samples (as illustrated in the sample preparation figure). A standard endodontic access cavity was prepared using a round diamond bur under air-rotor handpiece, and canal patency was confirmed using a size 10 K-file. The working length was established by inserting a size 10 K-file into the canal until it was visible at the apical foramen, and subtracting 0.5–1.0 mm from this measurement to standardize the working length across all specimens. All canals were

biomechanically prepared using a standardized instrumentation protocol up to a predetermined master apical file size, employing a step-back/crown-down technique as appropriate. During instrumentation, canals were irrigated with **3% sodium hypochlorite (NaOCl)** between each successive instrument change to remove organic debris and provide lubrication. Following completion of biomechanical preparation, canals were dried using sterile absorbent paper points. Samples were mounted on acrylic blocks to facilitate handling and irrigation using a negative-pressure endodontic irrigation system. Each group received its designated final irrigation protocol (Eco Enzyme, 17% EDTA, or normal saline) according to the contact times described below.

Final Irrigation Protocol

Following biomechanical preparation, specimens in both Group A (Smear Layer Removal) and Group B (Root Dentin Microhardness) were randomly and equally allocated to one of three subgroups based on the final irrigating solution used:

Group	Smear Layer Removal (Group A, n = 8)
Group 1	5 mL of Eco-enzyme
Group 2	5 mL of 17% EDTA
Group 3	5 mL of Normal saline

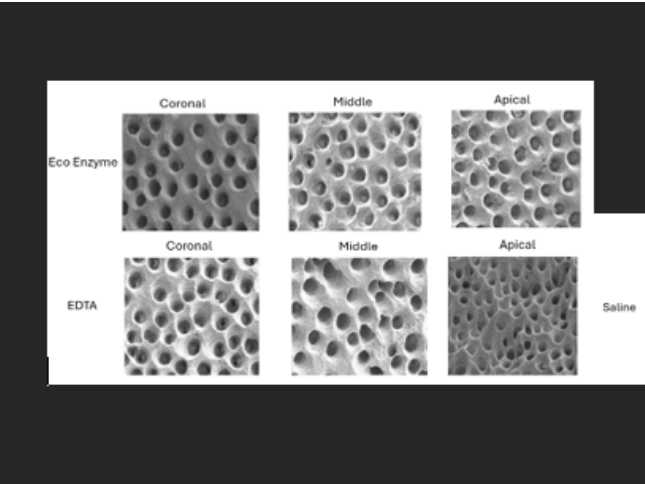
Evaluation of Smear Layer Removal (Group A)

Following irrigation, samples in Group A were prepared for scanning electron microscopic (SEM) analysis:

1. Samples were gold-sputter coated.
2. SEM imaging was performed at ×5000 magnification to evaluate the smear layer at the coronal, middle, and apical thirds of each root canal.
3. Smear layer was scored using a three-point scale:

Score	Criteria
Score	No smear layer — root

Score	Criteria
1	canal surface clean; all tubules open
Score	Moderate smear layer — root canal surface clean, but tubules contained debris
2	
Score	Heavy smear layer — smear layer covered both the root canal surface and the tubules
3	



Root Dentin Microhardness (Group B, n = 8)

Fig 2: SEM analysis of smear layering
Eco-enzyme applied for 5 minutes
removing ability of group1, group2, group3

17% EDTA Applied for 1 Minute

Normal saline applied for 1 minute

Samples in Group B were subjected to Vickers microhardness testing, with measurements recorded before and after treatment with the respective irrigant. The Vickers microhardness tester was selected over the Knoop hardness tester because it is better suited for evaluating surface changes in deeper dental hard tissue; the Knoop tester is more appropriate for superficial dentin (approximately 0.1 mm) rather than deeper dentin layers relevant to this study.

Statistical Analysis

Descriptive statistics (mean, standard deviation, minimum, and maximum scores) were calculated for the smear layer removal scores across the three irrigant groups at each root third (coronal, middle,

RESEARCH PAPER

apical). The Kruskal–Wallis test was used for intra-group comparison of irrigant efficacy across the coronal, middle, and apical thirds. The Mann–Whitney U test was used for intergroup comparison, specifically between the Eco Enzyme and EDTA groups, at each canal third.

Results

Root third	Ecoenzyme (Mean $\pm$ SD)	EDTA (Mean $\pm$ SD)	Normal saline (Mean $\pm$ SD)
Coronal third	1.25 $\pm$ 0.46	1.13 $\pm$ 0.35	1.88 $\pm$ 0.35
Middle third	1.38 $\pm$ 0.52	1.25 $\pm$ 0.46	1.88 $\pm$ 0.35
Apical third	1.38 $\pm$ 0.52	1.38 $\pm$ 0.52	2.50 $\pm$ 0.53

The distribution of scores among the three irrigating solutions—Ecoenzyme, EDTA, and Normal saline—was evaluated separately in the coronal, middle, and apical thirds. The mean scores and standard deviations for each group are presented in Table 1.

In the coronal third, the mean score was 1.25 $\pm$ 0.46 for the ecoenzyme group, 1.13 $\pm$ 0.35 for the EDTA group, and 1.88 $\pm$ 0.35 for the normal saline group. Kruskal–Wallis analysis demonstrated a statistically significant difference among the three irrigating solutions ($H = 10.17$, $df = 2$, $p = 0.006$).

In the middle third, the mean scores were 1.38 $\pm$ 0.52, 1.25 $\pm$ 0.46, and 1.88 $\pm$ 0.35 for ecoenzyme, EDTA, and normal saline, respectively. A statistically significant difference was observed among the three groups using the Kruskal–Wallis test ($H = 6.71$, $df = 2$, $p = 0.035$).

In the apical third, both ecoenzyme and EDTA demonstrated a mean score of 1.38 $\pm$ 0.52, whereas normal saline demonstrated a higher mean score of 2.50 $\pm$ 0.53. The difference among the three groups was statistically significant ($H = 11.90$, $df = 2$, $p =$

0.003).

Post hoc pairwise comparisons were performed using the Mann–Whitney U test with Bonferroni adjustment. In the coronal third, a statistically significant difference was observed between EDTA and normal saline (adjusted $p = 0.013$), whereas the differences between ecoenzyme and EDTA and between ecoenzyme and normal saline were not statistically significant. In the middle third, none of the pairwise comparisons remained statistically significant following Bonferroni adjustment. In the apical third, normal saline differed significantly from both ecoenzyme and EDTA (adjusted $p = 0.013$ for both comparisons), while no significant difference was observed between ecoenzyme and EDTA.

Overall, normal saline demonstrated higher scores than both ecoenzyme and EDTA, with the greatest difference observed in the apical third. Ecoenzyme and EDTA showed comparable scores across all three root thirds.

Table 1: Mean and Standard deviation

Root third	Kruskal–Wallis H	df	p-value	Interpretation
Coronal third	10.17	2	0.006	Significant
Middle third	6.71	2	0.035	Significant
Apical third	11.90	2	0.003	Significant

H = Kruskal–Wallis test statistic; df = degrees of freedom. $p < 0.05$ was considered statistically significant.

Table 2: Kruskal–Wallis test for intra-group comparison for efficacy of irrigants in smear layer removal in coronal, middle and apical third

Root third	Irrigant comparison	U	Adjusted p-value	Result
Coronal	Ecoenzyme	28.	1.000	NS

al	me vs EDTA	0		
	Ecoenzy me vs Normal saline	36. 0	1.000	NS
	EDTA vs Normal saline	8.0	0.013	Significa nt
Middl e	Ecoenzy me vs EDTA	28. 0	1.000	NS
	Ecoenzy me vs Normal saline	16. 0	0.158	NS
	EDTA vs Normal saline	16. 0	0.158	NS
Apical	Ecoenzy me vs EDTA	32. 0	1.000	NS
	Ecoenzy me vs Normal saline	6.0	0.013	Significa nt
	EDTA vs Normal saline	6.0	0.013	Significa nt

Table 3: Mann–Whitney U post-hoc table.

	Mean	Mean
	Pre	Post
Coronal	51.15 ± 1.1	46.05 ± 2.3
Middle	49.5 ± 2.15	42.65 ± 1.91
Apical	42.95 ± 0.59	38.25 ± 1.23

Table 4: Pre and Post-treatment Values of Vickers

Microhardness Measurement

Discussion

The present in vitro study evaluated the potential of fruit-peel-derived eco-enzyme as an alternative endodontic irrigant by assessing its smear-layer removal ability, effect on root dentin microhardness. The eco-enzyme was prepared from orange peel, lemon peel, jaggery and water in a 3:1:10 ratio and fermented for 3 months. The concept is particularly relevant because conventional irrigation protocols require agents with complementary properties, as NaOCl is primarily effective against organic tissue and microorganisms, whereas chelating agents such as EDTA facilitate removal of the inorganic component of the smear layer ^{12,13}.

In the present study, both eco-enzyme and 17% EDTA demonstrated better smear-layer removal than normal saline across all three root thirds. The mean smear-layer scores for eco-enzyme were **1.25 ± 0.46, 1.38 ± 0.52 and 1.38 ± 0.52** in the coronal, middle and apical thirds, respectively. Corresponding values for 17% EDTA were **1.13 ± 0.35, 1.25 ± 0.46 and 1.38 ± 0.52**, whereas normal saline showed higher scores of **1.88 ± 0.35, 1.88 ± 0.35 and 2.50 ± 0.53**, respectively. Since lower scores indicate better smear-layer removal, these findings demonstrate the superior cleaning ability of both eco-enzyme and EDTA compared with saline. These findings suggest that eco-enzyme possesses sufficient chemical activity to modify or remove the smear layer. Smear-layer removal is desirable because this layer may contain organic and inorganic debris, bacteria and bacterial by-products and can interfere with irrigant penetration and adaptation of root canal filling materials ^{12,13}.

The ability of eco-enzyme to remove the smear layer may be associated with its complex composition of fermentation-derived metabolites and bioactive compounds. Previous studies have demonstrated

biological activity of fruit-peel-derived eco-enzymes against endodontic microorganisms. Mavani et al. reported antimicrobial activity of fruit-peel eco-enzyme against *Enterococcus faecalis*, with concentrated preparations demonstrating activity comparable to 2.5% sodium hypochlorite under their experimental conditions ¹⁴. Sai et al. subsequently demonstrated substantial disruption of a multispecies endodontic biofilm following exposure to eco-enzyme ¹⁵. These findings support the potential of eco-enzyme as a biologically active endodontic solution, although its precise mechanism of smear-layer removal remains unclear. Unlike EDTA, which has a well-established calcium-chelating mechanism, eco-enzyme is a complex and non-standardized fermented preparation; therefore, the specific constituents responsible for its effect on dentin require further investigation.

he lower efficacy of all irrigants in the **apical third** observed in the present study may be attributed to the anatomical limitations of the apical region. Restricted canal dimensions, reduced irrigant exchange, limited accessibility and dentinal sclerosis can interfere with effective irrigant penetration and smear-layer removal. Similar limitations have been reported with conventional chelating agents, with cleaning generally being more predictable in the coronal and middle thirds than in the apical region ^{12,16}. The superior apical performance of EDTA in the present study may therefore be related to its established and predictable calcium-chelating ability.

An important finding was that **eco-enzyme produced a significantly lesser reduction in root dentin microhardness than 17% EDTA**. This finding is clinically relevant because excessive demineralization may adversely affect the mechanical properties of radicular dentin. EDTA removes calcium ions through chelation and prolonged exposure may result in dentinal erosion

and reduction in microhardness ^{16,17}. Previous studies have also demonstrated that EDTA can produce greater microhardness reduction than several alternative irrigating solutions ^{17,18}. Thus, the comparatively smaller reduction produced by eco-enzyme may indicate a less aggressive interaction with the mineralized dentinal substrate.

However, this finding should be interpreted in relation to the **different contact times used in the study**. EDTA was applied for 1 minute, whereas eco-enzyme was applied for 5 minutes. The 5-minute exposure for eco-enzyme was selected based on previously reported antimicrobial activity, in which substantial biofilm disruption was observed after 5 minutes ¹⁵. Therefore, direct comparison of the two agents should be interpreted cautiously, and future investigations should evaluate both irrigants using standardized contact times and different concentrations.

The present findings therefore indicate that eco-enzyme may have a dual advantage of effective smear-layer removal with comparatively less alteration of dentin microhardness. Similar approaches have been investigated with other alternative chelating agents, where the objective has been to achieve adequate smear-layer removal while minimizing dentinal demineralization ^{18,19}.

The concept is particularly important because an ideal final irrigant should effectively remove the smear layer without causing excessive alteration of the underlying dentin. The use of eco-enzyme also introduces an environmentally sustainable perspective because it utilizes fruit-peel waste as the raw material. Previous investigations have demonstrated that fermentation of fruit peels can produce preparations with antimicrobial activity, supporting their potential application in dentistry ^{14,15}.

The present study extends this concept by demonstrating potential effects on the dentinal

substrate in addition to previously reported antimicrobial properties.

Nevertheless, the results should be interpreted within the limitations of the study. Eco-enzyme is a **non-standardized biological preparation**, and its composition may vary according to the type of fruit peel, fermentation period, sugar concentration and preparation conditions. The present study evaluated only one formulation—orange and lemon peel with jaggery fermented for 3 months. Furthermore, the study did not chemically characterize the individual constituents responsible for its smear-layer-removing activity. The unequal exposure periods between eco-enzyme and EDTA and the in vitro nature of the experiment should also be considered when extrapolating the findings to clinical practice. Overall, the findings suggest that **fruit-peel-derived eco-enzyme is a promising experimental endodontic irrigant**, demonstrating smear-layer removal comparable to 17% EDTA and relatively better preservation of root dentin microhardness. Further studies should focus on chemical standardization, optimization of concentration and contact time, antimicrobial and tissue-dissolving efficacy, dentinal erosion, cytotoxicity and biocompatibility before clinical application.

Conclusion:

Within the limitations of the present in vitro study, fruit-peel-derived eco-enzyme demonstrated promising potential as an endodontic irrigant. Eco-enzyme and 17% EDTA showed better smear-layer removal than normal saline across the coronal, middle and apical thirds, with comparable mean smear-layer scores in the apical third. Eco-enzyme also produced a lesser reduction in root dentin microhardness compared with 17% EDTA, suggesting a more conservative effect on dentin. Furthermore, unlike EDTA, the addition of eco-enzyme to sodium hypochlorite did not result in a significant reduction in free available chlorine,

indicating potential chemical compatibility under the conditions tested. Therefore, eco-enzyme may represent a promising and environmentally sustainable alternative or adjunct to conventional endodontic irrigants; however, further studies are required to establish its optimal concentration, contact time, antimicrobial and tissue-dissolving efficacy, chemical composition, and biocompatibility before clinical application.

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