Original Research

https://doi.org/10.36377/ET-0133

The effect of C³Mix: An experimental antimicrobial root canal irrigant on removal of smear layer and its depth of penetration into the dentinal tubules

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Abstract

AIM of this in vitro study was to compare and evaluate the antimicrobial efficacy, smear layer removal, depth of penetration and tissue dissolving capacity of C³Mix.

MATERIALS AND METHODS. 36 single-rooted, single-canal teeth were divided in 3 groups (n = 12 each) and irrigated with Saline, Ethylene diamine tetra-acetic acid (EDTA) and C³Mix. The teeth were observed under Scanning electron microscope (SEM) to evaluate smear layer removal and Confocal laser scanning microscope (CLSM) to check for depth of penetration of the irrigant. Disc diffusion tests were performed in triplicate manner with 2 control groups to assess and compare the antimicrobial efficacy of irrigants against E faecalis. Tissue dissolution in C³Mix, NaOCl and CHX solutions were performed using bovine dental pulp immersion in test solutions for 5 minutes and removing after 5 minutes to check for weight loss. The results were statistically analysed.

RESULTS. C³Mix was statistically more effective than EDTA and Saline in removing smear layer and increasing the depth of penetration ($p < 0.05$) in dentin. NaOCl was significantly significant than that of C³ Mix in antimicrobial activity. NaOCl showed better tissue dissolving capacity when compared with C³Mix.

CONCLUSIONS. C³Mix proves to be a good antimicrobial agent and has superior smear layer removal and increased depth of penetration in comparison with EDTA but has reduced tissue dissolving capacity than NaOCl.

Keywords: ethylene diamine tetra-acetic acid, C³Mix, antimicrobial efficacy, smear layer, depth of penetration, chemical interaction

Article info: received – 15.09.2025; revised – 20.10.2025; accepted – 28.10.2025

Conflict of interest: The authors report no conflict of interest.

Acknowledgements: There are no funding and individual acknowledgments to declare.

For citation: Thopate N.T., Chakravarthy D., Ramasamy R., Raja V., Lakshmi S., Dev D. The effect of C³Mix: An experimental antimicrobial root canal irrigant on removal of smear layer and its depth of penetration into the dentinal tubules. *Endodontics Today.* 2025;23(4):584–591. https://doi.org/10.36377/ET-0133

Влияние C³Mix: экспериментального антимикробного ирриганта для корневых каналов на удаление смазочного слоя и глубину его проникновения в дентинные трубочки

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Резюме

ЦЕЛЬ ИССЛЕДОВАНИЯ. Целью данного *in vitro* исследования было сравнение и оценка антибактериальной эффективности, удаления смазочного слоя, глубины проникновения и способности растворять ткани раствора C³Mix.

МАТЕРИАЛЫ И МЕТОДЫ. Использовано 36 однокорневых зубов с одним каналом, разделённых на 3 группы (по 12 зубов в каждой) и орошаемых растворами: физиологический раствор, Этилендиаминтетрауксусная кислота (EDTA) и C³Mix. Зубы исследовали с помощью сканирующего электронного микроскопа (SEM) для оценки удаления смазочного слоя и конфокальной лазерной сканирующей микроскопии (CLSM) для определения глубины проникновения ирриганта. Антибактериальная эффективность оценивалась методом дисковой диффузии в трипликате с двумя контрольными группами против *Enterococcus faecalis*. Способность к растворению тканей оценивалась на образцах бычьей пульпы с погружением в растворы C³Mix, NaOCl и CHX на 5 минут с последующим взвешиванием для определения потери массы. Результаты подвергались статистическому анализу.

РЕЗУЛЬТАТЫ. Раствор C³Mix статистически превосходил EDTA и физиологический раствор по эффективности удаления смазочного слоя и глубине проникновения в дентин ($p < 0,05$). NaOCl показал зна-

чительно более высокую антибактериальную активность по сравнению с C³Mix. Также NaOCl продемонстрировал лучшую способность к растворению тканей по сравнению с C³Mix.
ВЫВОДЫ. C³Mix является эффективным антибактериальным средством, превосходит EDTA по удалению смазочного слоя и глубине проникновения, но уступает NaOCl в способности к растворению тканей.

Ключевые слова: C³Mix, удаление смазочного слоя, антибактериальная активность, глубина проникновения, растворение тканей

Информация о статье: поступила – 15.09.2025; исправлена – 20.10.2025; принята – 28.10.2025

Конфликт интересов: Авторы сообщают об отсутствии конфликта интересов.

Благодарности: Финансирование и индивидуальные благодарности для декларирования отсутствуют.

Для цитирования: Топате Н.Т., Чакраварти Д., Рамасами Р., Раджа В., Лакшми С., Дев Д. Влияние C³Mix: экспериментального антимикробного ирриганта для корневых каналов на удаление смазочного слоя и глубину его проникновения в дентинные трубочки. *Эндодонтия Today.* 2025;23(4):584–591. https://doi.org/10.36377/ET-0133

INTRODUCTION

The main challenge in endodontic therapy is to completely disinfect the root canal system as it has many intricacies such as lateral canals, accessory canals, fins, isthmuses and apical delta. Biomechanical preparation of the root canals can lead to accumulation of organic and inorganic debris. An ideal irrigant should efficiently eliminate the micro-organisms, organic and inorganic debris while maintaining an ideal surface contact with the root canal [1; 2].

There is no ideal irrigant that can fulfil the above-mentioned properties. The irrigant that shows closet proximity to the above-mentioned properties are MTAD and QMix. The disadvantages of MTAD includes discoloration and less shelf life i.e., it needs to be prepared freshly. QMix leads to formation of orange brown precipitate when used after NaOCl [3].

The gold standard irrigant in removing organic debris is Sodium hypochlorite which is also an effective antimicrobial agent but it has certain disadvantages such as it lacks substantivity and its disability to remove inorganic debris [2; 3]. To eliminate inorganic debris from the root canal system formed from bio-mechanical preparation, many studies proved citric acid as a better chelating agent than EDTA [4–6]. Chlorhexidine (CHX) is a cationic bisbiguanide which is most effective against E faecalis. It is the broad spectrum antimicrobial agent and also has substantivity [7; 8].

Cetyl pyridinium chloride (CPC) is a cationic surfactant. The addition of surfactant facilitates the contact of the irrigant to the dentinal surface, thereby improving the wettability and deeper penetrability of irrigant into dentinal tubules [9].

Thus the goal of this study is to identify an appropriate irrigating solution that can fulfil all the above requirements mentioned [10; 11]. The aim of this in vitro study was to compare and evaluate the antimicrobial efficacy, smear layer removal, depth of penetration and tissue dissolving capacity of C³Mix.

MATERIALS AND METHODS

Ethical approval

The study protocol was approved by the Institutional Ethics Committee. In this study, the principles of the Declaration of Helsinki and its later amendments or comparable ethical standards were followed.

Preparation of C³mix (Chlorhexidine + Citric Acid + Cetyl Pyridinium Chloride)

2% Chlorhexidine+ 10% Citric Acid+ 0.1% Cetyl Pyridinium Chloride were mixed to formulate C³Mix in the ratios of 1:0.5:1. These samples were characterized using UV Visible spectroscopy (JASCO UV VIS) and Fourier Transform Infrared Spectroscopy (BRUKER, USA). UV visible spectroscopy and Fourier Transform Infrared Spectrometry (FTIR) to check if it is a stable complex and there is a successful integration of the three components and their synergistic effects in the formulation (Fig. 1).

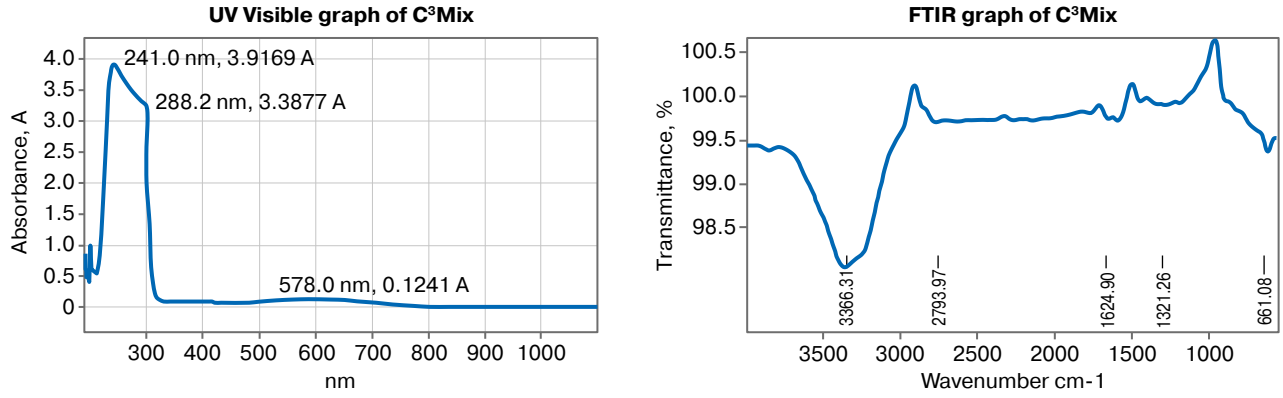

Fig. 1. Spectroscopic characterization of the C³Mix solution (UV-Vis and FTIR analysis)

Рис. 1. Спектроскопическая характеристика раствора C³Mix (UV-Vis и FTIR-анализ)

Assessment of antimicrobial efficacy

Agar Disc Diffusion method was used in triplicate method for assessment of antimicrobial property of C^3Mix, NaOCl and saline against E. *faecalis* (ATCC 29212). The inhibition zones were measured in millimeters after incubation at 37°C for 24 hours [6].

Assessment of smear layer removal and depth of penetration

36 human anterior teeth with single-root and single-canal with similar root canal length, were included and divided into three groups (n = 12) for study. Permanent teeth with intact apices, without previous root canal treatment or restorations were included. Tooth with cracks, root caries, fractures, external or internal root resorption, calcification and apical diameters larger than size .30 were excluded. The teeth were decoronated and standardized to a 15 mm length using a diamond disc in a slow speed micromotor handpiece (NSK Ltd, Tokyo, Japan).

The canals were negotiated with #10, and #15 K files (MANI Inc, Tochigi, Japan) and canal preparation was done upto 45/.05 using Reciprocating WAVE ONE GOLD (large Size- Dentsply). Samples were irrigated with 1 ml/min of 5.25% NaOCl as a primary irrigating solution using a disposable 2 ml syringe with 30-gauge needle during instrumentation up to 1 mm short of the working length. Irrigation was done with saline to eliminate NaOCl effectively [6]. Samples were divided in 3 groups (n = 36) as:

– Group 1: 5 ml of Saline (n = 12) irrigation for 5 min;
– Group 2: 5 ml of EDTA (n = 12) irrigation for 5 min;
– Group 3: 5 ml of C^3Mix (n = 12) irrigation for 5 min.

Smear layer removal analysis by scanning electron microscope

Longitudinal grooves were created along the entire root lengthwise to create a buccolingual split along the long axis to expose the entire extent of the root canal. The samples were dehydrated and sputter coated with a gold layer for scanning electron microscope (CARL ZEISS, USA) analysis. For every specimen imaging was done at coronal, middle and apical third and examined by two blinded and calibrated examiners to evaluate smear layer removal [6] and evaluated by Hülsmann scoring system [12].

Score 1: No smear layer, dentinal tubules visualized.

Score 2: Small amount of smear layer, many dentinal tubules visualized.

Score 3: Smear layer and debris covering the root canal walls, a few dentinal tubules visualized.

Score 4: The surface of root canal covered completely with smear layer; no dentinal tubules visualized.

Score 5: Heavy smear layer and debris covered the root canal surface.

Depth of penetration assessed by confocal laser scanning microscope

The samples were stained using rhodamine B dye (RESEARCH LAB FINE CHEM INDUSTRIES, INDIA). The samples were sectioned across root lengths at 2 mm, 5 mm, and 8 mm from apex to check for the depth of penetration in the coronal, middle and apical third of the root canal. The slices were mounted and observed under confocal laser scanning microscope (WHITE LASER) to evaluvate depth of penetration [13].

Assessment of tissue dissolving capacity

The bovine teeth were horizontally cut at the cemento-enamel junction to separate the crown and root portions using diamond bur. Bovine Pulp tissue was removed from the root canal with fine-tipped forceps and any remnant blood or debris were washed away with distilled water. The samples were then dried with paper towels and weighed on a precision scale. The pulp was segregated into pieces weighing approximately 25±5 mg with the help of a #12 scalpel. These tissues were immersed in NaOCl, CHX and C^3Mix and kept for 5minutes. The positive control used was NaOCl and the positive control used was chlorhexidine. After 5 minutes the pulp tissues were removed and blot dried and weighted again on precision scale.The initial and final measurements was calculated and the difference were statistically analyzed [14].

Statistical analysis

Statistical analysis for the antimicrobial activity and tissue dissolution capacity was performed by taking the mean and standard deviation. The data of debris scores and smear layer scores were analysed using the Chi square test to verify the categorical outcomes. For Depth of penetration, ANOVA was used to analyse the data. Descriptive statistics was done to assess the mean among the study variables. To analyse the mean difference of irrigants for depth of penetration, Post Hoc test was performed using SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp. Released 2019). Significance level was fixed as 5% (α = 0.05). P-value < 0.05 was considered to be statistically significant.

RESULTS

Anti-microbial efficacy

For *E. faecalis* (Fig. 2), the inhibition zone provided by NaOCl was significantly larger than C^3Mix. Statistical analysis showed that the inhibition zone provided by NaOCl had a significantly (p > 0.05) larger diameter (30 mm) of inhibition zone than C^3Mix (15 mm) and Saline (6 mm).

Table 1. Mean and standard deviation of disc diffusion test done in triplicate format

Таблица 1. Средние значения и стандартное отклонение результатов теста дисковой диффузии, выполненного в трипликате

Samples	MEAN ± SD
SALINE	6±0
C^3Mix	15±0
NAOCL	30±0

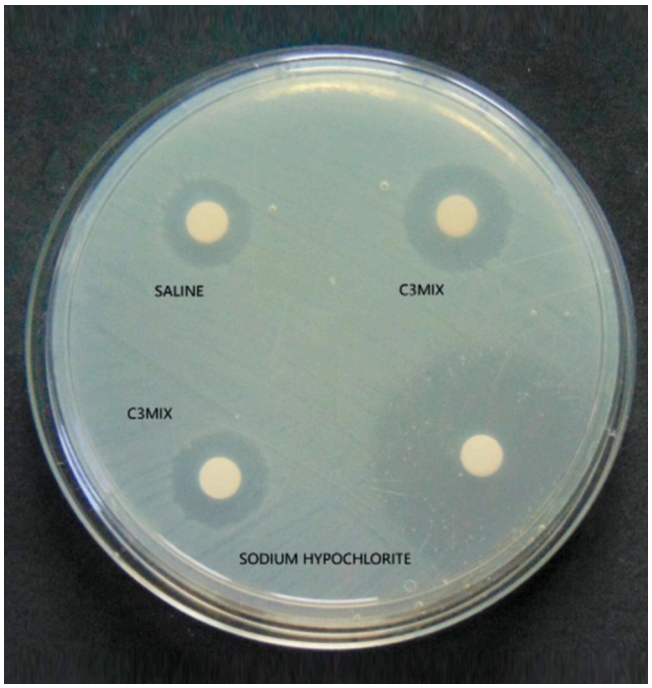

Fig. 2. Inhibition zones of *E. faecalis* growth after exposure to NaOCl, C³Mix, and saline

Рис. 2. Зоны ингибирования роста *E. faecalis* при воздействии NaOCl, C³Mix и физиологического раствора

Smear layer removal evaluation

SEM images taken at the coronal, middle, and apical third of the root canal wall are shown in Fig. 3. The dentinal tubules in the control group were completely covered with a smear layer in all thirds of the root canal wall. In the EDTA group the tubules were mostly visible at the coronal and middle third of the root canal wall. In C³Mix group the tubules were visible at the coronal and middle third and apical third of the root canal wall.

The results for the smear layer removal are shown in Table 2. C³Mix was statistically more effective than NaOCl / EDTA and saline in removing smear layer from all root canal thirds ($p < 0.05$). There was a significant difference between NaOCl / EDTA and C³Mix only in the removal of debris from the apical third ($p < 0.05$).

Table. 2. Mean score registered for each group in the different root canal thirds in evaluating residual smear layer Smear

Таблица 2. Средние показатели, зарегистрированные для каждой группы в различных третях корневого канала при оценке остаточного смазочного слоя

Smear layer	Saline (mean ± SD)	EDTA (mean ± SD)	C³Mix (mean ± SD)
Coronal third	4.3 ± 0.5	2.1 ± 0.4	2.0 ± 0.4
Middle third	4.6 ± 0.3	2.9 ± 0.6	2.2 ± 0.5
Apical third	5.0 ± 0.0	3.2 ± 0.7	1.9 ± 0.3

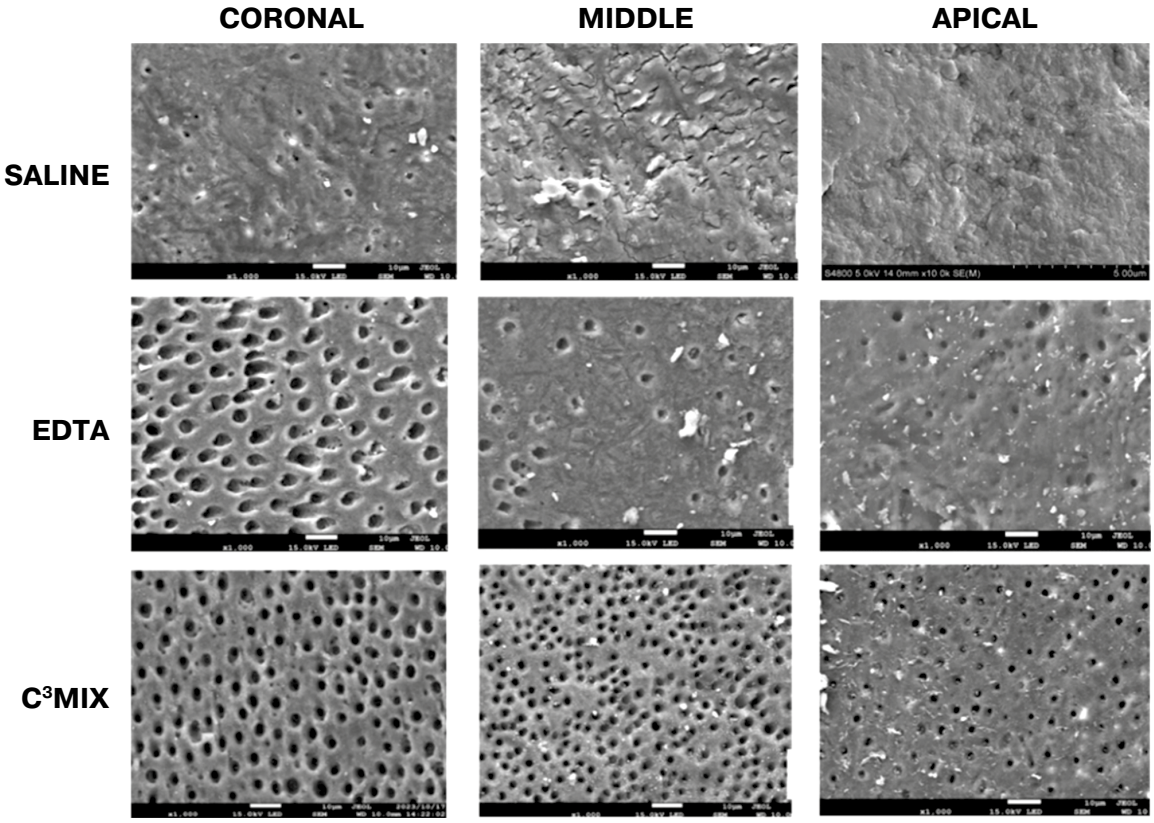

Fig. 3. SEM images of the root canal walls at the coronal, middle, and apical thirds after irrigation with different solutions (control, EDTA, C³Mix)

Рис. 3. СЭМ-изображения стенок корневого канала в коронковой, средней и апикальной третях после обработки различными ирригантами (контроль, EDTA, C³Mix)

Evaluation of depth of penetration

CLSM images representing the coronal, middle, and apical third of the root canal walls are shown in Fig. 4.

The control group showed minimal penetration into dentinal tubules at all thirds of the root canal wall. Penetration into dentinal tubules in EDTA group was highest in coronal third compared to that of middle and apical. C^3Mix shows highest penetration in dentinal tubules at the coronal, middle and apical third of the root canal wall compared to that of EDTA (Table 3).

Table 3. Mean score registered for each group in the different root canal thirds in evaluating depth of penetration

Таблица 3. Средние показатели, зарегистрированные для каждой группы в различных третьях корневого канала при оценке глубины проникновения

Depth of penetration	Saline (mean ± SD)	EDTA (mean ± SD)	C^3Mix (mean ± SD)
Coronal third	177.5±83.90	2,012.58±185.27	2,222.70±156.77
Middle third	109.89±34.65	1,487.27±150.58	1,932.38±161.67
Apical third	75.918±17.282	644.94±168.70	1,441.63±219.73

Evaluation of tissue dissolving capacity of C^3Mix

Tissue dissolution was seen more in NaOCl while C^3Mix showed mild tissue dissolution and CHX shows no tissue dissolution capacity (Table 4).

Table 4. Initial and Final measurement of pulp tissue after inserting in solutions

Таблица 4. Первоначальные и конечные значения массы пульпы после погружения в растворы

Groups	Initial (Mean ± SD)	Final (Mean ± SD)
NaOCl	25.40±1.00	12.40±1.00
C^3Mix	22.39±0.53	21.39±0.72
CHX	25.83±1.00	25.78±0.90

DISCUSSION

Irrigation is considered as the primary mode of cleaning and disinfecting of the root canal system [1]. Elimination of smear layer during biomechanical preparation of the root canal system is one of the main challenges for an irrigant [10]. No single irrigant can efficiently eliminate smear layer while maintaining an ideal surface contact with the root canal [13].

This study focuses on evaluating antimicrobial efficacy, smear layer removal, depth of penetration, tissue dissolving capacity of an experimental irrigant- C^3Mix [9].

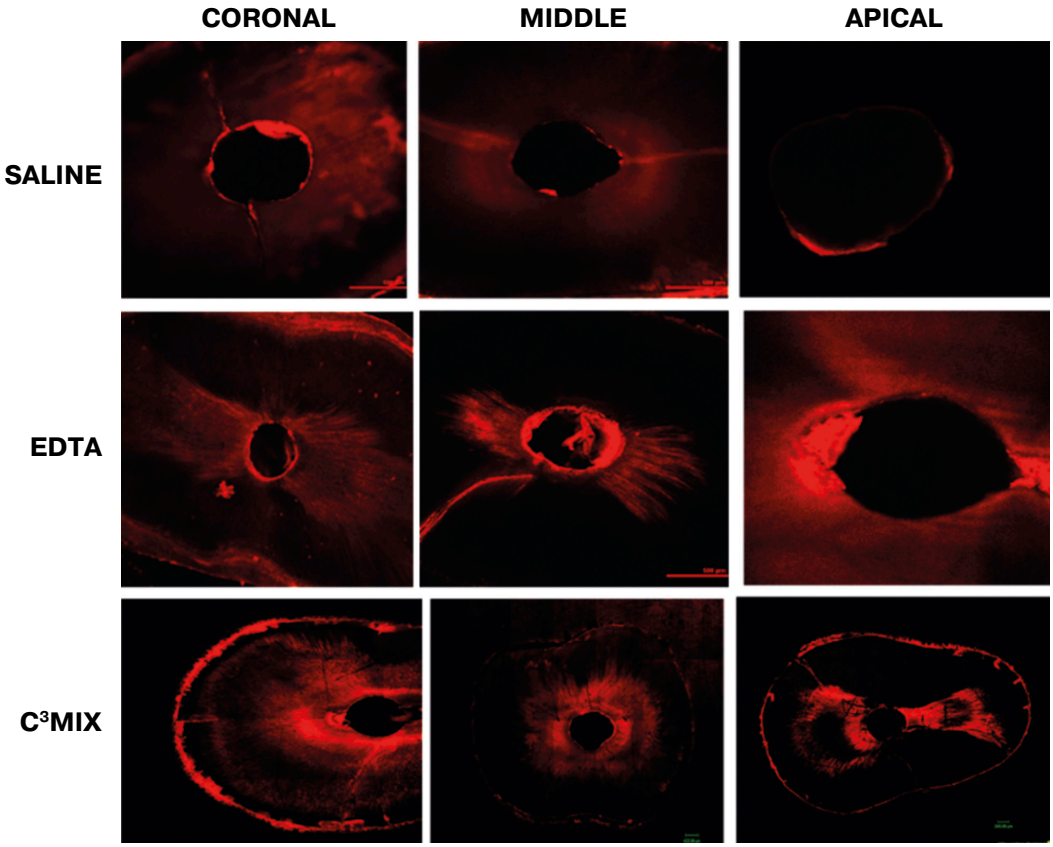

Fig. 4. CLSM images of the root canal walls at the coronal, middle, and apical thirds after irrigation with different solutions (control, EDTA, C^3Mix)

Рис. 4. CLSM-изображения стенок корневого канала в коронковой, средней и апикальной третях после обработки различными ирригантами (контроль, EDTA, C^3Mix)

2% Chlorhexidine+ 10% Citric Acid+ 0.1% Cetyl Pyridinium Chloride added to formulate C^3Mix in the ratios of (1:0.5:1) exhibited more stable complex and successful integration of the three components and their synergistic effects in the formulation enhancing its antimicrobial efficacy (Fig. 1).

The present study compared the antimicrobial efficiency of C^3Mix with Sodium hypochlorite (positive control) and Saline (negative control) against *E. faecalis.* [6; 14]. The results concluded that NaOCl showed higher antimicrobial efficacy than C^3Mix. Research suggests that Sodium hypochlorite showed highest antimicrobial activity as it is a strong oxidizing agent [10; 14; 15]. NaOCl when comes in contact with bacterial cells, it reacts and oxidizes essential proteins and enzymes, leading to their denaturation and inactivation. It releases hydroxyl ions (OH-) which increases the pH and disrupts bacterial cell membrane and inhibits essential enzymatic reactions. It degrades fatty acids and lipids which results in the formation of soap and glycerol and can form chloramines that interfere with bacterial cell metabolism and cause irreversible enzymatic inactivation [1; 2; 10]. The antibacterial efficacy of C^3Mix is due to presence of Chlorhexidine which is a proven antibacterial and antifungal agent and Cetyl Pyridinium Chloride, an effective antibacterial and antiviral agent.

The second part of the study aimed at evaluating smear layer removal from dentinal tubules. According to the above-mentioned criteria, C^3Mix was most efficient in smear layer removal from coronal, middle and apical followed by EDTA and least in Saline. This smear layer removal property of C^3Mix is due to Citric acid present in it [16; 17]. Haznedaroglu also reported that the irrigant solution with lower pH showed greater smear layer removal ability [18; 19]. Citric acidhas pH around 1.4 while the pH of EDTA is around 4.5 to 5.5 [20; 21]. In addition, the other ingredient of C^3Mix, Cetyl Pyridinium Chloride which maintains intimate contact with root surface, removes smear layer efficiently [9].

The third part of the study was aimed to evaluate the depth of penetration of irrigants into the dentinal tubules. In this study, we found the depth of penetration of the C^3Mix to be pronounced and statistically significant in the coronal third followed by the middle and the apical third as Cetyl Pyridinium Chloride is a surfactant which increases the wettability and increases the penetrability of irrigant inside the dentinal tubules [22]. This is in agreement with the previous study conducted by Mukhlif and Al-Hashimi in 2021 that irrigant with surfactant showed better penetration into the dentinal tubules than the irrigant without the surfactant, which proves that the surfactant in the composition leading to the higher penetration [22; 23].

The penetration of irrigants seen in the apical third was less due to lower patent tubules in the apical region because of dentinal sclerosis and insufficient access to this region for irrigants to flush out the debris and remove the smear layer to maintain the patency of the tubules [22].

Lastly, the tissue dissolution capacity was checked among C^3Mix, NaOCl and CHX. NaOCl was used as positive control and CHX as negative. The results concluded that tissue dissolving capacity of NaOCl was highest as it takes place in 3 steps-saponification, amino acid neutralization and chloramination reaction, while C^3Mix lacks strong oxidizing agent and shows mild tissue dissolution due to presence of citric acid [1; 10]. No tissue dissolving capacity was seen in CHX as suggested by previous studies.

The limitations of this present study are – Root canal infection is poly-microbial. Therefore, antimicrobial study in the form of multi-species may be a better replacement for the true working environment of the root canal system. The study was based on freshly mixed C^3Mix Further shelf life of the sample needs to be assessed. Cell toxicity should be studied. No added activation methods were attempted to facilitate the depth of penetration. The chemical precipitate formed during interaction of C^3Mix with NaOCl is not specified.

CONCLUSION

C^3Mix is a promising, cost-effective root canal irrigant with significant smear layer removing capability and antimicrobial activity with substantivity property. Its performance exceeds EDTA in smear layer removal and depth of penetration. Further studies are necessary to validate its clinical applicability and long-term effects on various biofilm models.

HIGHLIGHTS

1. C^3 Mix shows effective removal of smear layer from dentinal tubules in coronal, middle and apical third when compared to 17% EDTA.

2. The depth of penetration of irrigants into dentinal tubules was seen higher in C^3 Mix in coronal, middle as well as in apical third of root canals when compared to 17% EDTA.

3. NaOCl shows higher antimicrobial activity and tissue dissolving capacity than C^3 Mix.

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AUTHOR'S CONTRIBUTION

All the authors made equal contributions to the publication preparation in terms of the idea and design of the article; data collection; critical revision of the article in terms of significant intellectual content and final approval of the version of the article for publication.

ВКЛАД АВТОРОВ

Все авторы внесли равноценный вклад в подготовку публикации в части замысла и дизайна исследования; сбора данных; критического пересмотра статьи в части значимого интеллектуального содержания и окончательного одобрения варианта статьи для опубликования.