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Original research

The impact of maleic acid, carbohydrate-derived fulvic acid, and ethylenediaminetetraacetic acid on the flexural strength and modulus of elasticity of human radicular dentin: an *in vitro* study

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Purpose

This study aimed to evaluate the impact of ethylenediamine tetra-acetic acid (EDTA), carbohydrate-derived fulvic acid (CHD-FA), and maleic acid (MA) on the flexural strength (FS) and modulus of elasticity (ME) of human radicular dentin.

Materials and Methods

Fifteen freshly extracted human mandibular premolars with single roots and single canals were selected. The teeth were decoronated 2 mm below the cementoenamel junction (CEJ), and the roots were sectioned into buccal and lingual halves. Each half was used to prepare two standardized plano-parallel dentin bars (7 mm in length × 1 mm in width × 1 mm in height). The samples were then divided into four groups and immersed in their respective irrigating solutions for 5 minutes: saline (control), maleic acid, CHD-FA, and EDTA. Subsequently, FS and ME were evaluated using a three-point bending test.

Results

Both EDTA and maleic acid reduced the FS and ME of radicular dentin. However, the study demonstrated that higher mean values for FS and ME were recorded in the CHD-FA and saline (control) groups compared to the EDTA and maleic acid groups.

Conclusion

CHD-FA is recommended as an endodontic irrigant, as it does not compromise the mechanical properties of radicular dentin, specifically FS and ME.

Keywords: *Radicular dentin, flexural strength, modulus of elasticity, EDTA, CHD-FA, maleic acid*

Introduction

Proper sequential irrigation in addition to mechanical instrumentation exhibits a synergistic effect on the success of endodontic treatment. Saleh *et al.* (1) illustrated the significance of eliminating the smear layer and the presence of open dentinal tubules in facilitating the disinfecting effect of intracanal medicaments. In the absence of bacterial infection, pulpal and peri-radicular pathosis do not develop (2). However, the mechanical and structural properties of the radicular dentin are altered by the use of various irrigating solutions.

An ideal root-canal irrigant should effectively disinfect the complete root-canal system, eliminate the smear layer, and enable the infiltration of antimicrobial agents into the dentinal tubule, ensuring a long-lasting antibacterial effect. Since all these properties cannot be achieved by using a single irrigating solution, the sequential use of multiple irrigants is

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required to achieve the same. Maintaining the physical and mechanical characteristics of exposed dentin is crucial, and these solutions should not cause any changes to these properties. It should neither discolour the tooth nor interfere with the sealing ability of the obturation materials (3).

The presence of a smear layer, which forms over dentinal tubules following mechanical instrumentation, can hinder the disinfection process. Eventually, this smear layer is broken down by the proteolytic enzymes of bacteria, resulting in microleakage and an imperfect seal (4). Thus, the removal of this layer enhances the adherence of the obturating materials to the canal walls. Chelating agents are preferred for this process because they create a complex with the calcium ions in the dentin, making it more fragile and facilitating easier instrumentation.

The most commonly used irrigants for endodontic treatment are sodium hypochlorite (NaOCl), Ethylenediamine tetra-acetic acid (EDTA), Chlorhexidine gluconate (CHX) and saline. Each has its advantages and disadvantages. NaOCl has a potent antimicrobial effect and tissue dissolving capacity, but it does not remove the smear layer and negatively influences the modulus of elasticity (ME) and flexural strength (FS) of dentine (5,6). Smear layer is efficaciously removed with EDTA and when employed as a final irrigant, it unclogs up the dentin which allows the obturating material to even fill the lateral canals (7). The wettability of an irrigating solution is improved with surfactant, which reduces surface tension and contact angle to the dentinal walls (8). In this study Smear Clear (Sybron Endo, Orange, CA) was used which contains 17% EDTA, cetrimide, and two other surfactants (poly-oxyethylene and iso-octyl-cyclohexyl). It reduces surface tension and is efficient in eliminating the smear layer in all thirds of the root when compared to 17% EDTA (9).

In adhesive dentistry, MA is applied as an acid conditioner to get rid of the smear layer from the tooth surface. Previous in-vitro research has shown that 7 % MA was more potent in smear layer removal from the apical third compared to 17% EDTA solution. MA improved the seepage of the irrigant and sealer through the tubules creating more resin tags. On the contrary, the demineralizing effect of MA causes a downside in the mineral content of the peri and inter-tubular dentin along with its influence on the organic materials in dentine (10).

CHD-FA has been found to have antibacterial and antifungal properties, making it a promising new antimicrobial agent (11,12). Furthermore, it has been reported that it is non-toxic and possesses anti-inflammatory properties (13). It also has antioxidant properties due to the presence of a phenolic hydroxyl group and metal-chelating ability (14). A recent study showed significant smear layer removal with minimal reduction in microhardness (15).

While evaluating the microhardness of an irrigant on root dentin provides valuable insights into surface resistance, conducting subsequent analyses on flexural strength and modulus of elasticity is imperative. These additional tests offer a comprehensive understanding of the structural integrity and overall mechanical behaviour of the dentin, allowing for a more detailed assessment of the irrigant's impact on the dentin's load-bearing capacity and flexibility. The present study investigated the effect of root canal irrigating solutions like MA, CHD-FA, and EDTA on the FS and ME of the radicular dentin. We formulated the following null hypothesis: there is no significant difference in the smear layer removal and mechanical properties of dentin when using CHD-FA compared to other standard irrigants.

Materials and Methods

Ethical statement

The protocol for this in vitro study was approved by the Institutional Ethical Committee of Sree Balaji Dental College & Hospital, Bharath Institute of Higher Education and Research (Approval number: SBDCH/IEC/12/2020/25).

Sample size estimation

Before initiating the main study, a pilot study was conducted to assess feasibility and refine the methodology. This pilot study involved five samples per group, prepared by decoronating five freshly extracted single-rooted premolars. The objective was to ensure the appropriateness of the experimental procedures and validate the protocols for measuring the flexural strength (FS) and modulus of elasticity (ME) of dentin bars (16). The minimum sample size required for the study was determined using the G*Power software (Version 3.1.9.4), based on the means and standard deviations of FS and ME obtained from the pilot study. Since four groups were compared, a one-way analysis of variance (ANOVA) was used to calculate the effect sizes based on the means and standard deviations of the groups. The results of the sample size calculation are summarized in Table 1. Since the minimum sample size required for the study ranged between four and 13, it was decided to include 15 samples per group (n=15), resulting in a total of sixty samples across all four groups (N=60).

Table 1. *Sample size calculation protocol*

	Based on Maximum SD					Based on Minimum SD				
	Effect Size	Total Sample size	Sample size for each group	α err prob	Actual Power (1-β err prob)	Effect Size	Total Sample size	Sample size for each group	α err prob	Actual Power (1-β err prob)
FS	.7073	40	10	0.05	.9583	1.2609	16	4	0.05	.9599
ME	.6044	52	13	0.05	0.9538	0.7395	36	9	0.05	.9528

FS (Flexural strength); ME (Modulus of Elasticity)

Sample preparation

For the main study, fifteen freshly extracted single-rooted premolars, obtained for orthodontic purposes, were immediately stored in distilled water after extraction and maintained at a room temperature of 37°C in 100% humidity. The teeth were decoronated 2 mm apical to the cementoenamel junction (CEJ) using a diamond saw. The root canals were instrumented using No. 10 and No. 15 stainless steel K-files (Mani, Japan) following the determination of the working length. After establishing a glide path, further canal preparation was performed using ProTaper NiTi Universal Rotary Files (Dentsply, Maillefer, USA) up to size F3. During the preparation, 1 ml of saline was used between each instrumentation step. No other irrigating solution was used during or after canal preparation. Following instrumentation, the canals were sealed with a sterile cotton pellet. Two vertical grooves, each 2 mm in depth, were created on the buccal and lingual root surfaces using a diamond saw. The roots were then split longitudinally using the cut-and-split method with a chisel and mallet. Standardized plano-parallel dentin bars (1 mm × 1 mm × 7 mm) were prepared from the cervical half of the root using a diamond saw. Two dentin bars were obtained from one-half of the split root, yielding a total of four plano-parallel dentin bars per root. The prepared dentin bars were stored in saline at 37°C in four separate airtight containers.

Test groups

The dentin bars from each container were randomly assigned to one of four different irrigation solutions. Group 1 was treated with normal saline, Group 2 with 7% maleic acid (MA), Group 3 with 5% carbohydrate-derived fulvic acid (CHD-FA), and Group 4 with 17% EDTA (Smear Clear) (SybronEndo, Orange, CA). These solutions were applied to the dentin bars for five minutes, ensuring full immersion. Following treatment, the test solutions were neutralized, and the bars were stored in saline.

Three-point bending test

The three-point bending test, conducted using a test jig mounted on the Universal Testing Machine (UTM), was a crucial step in evaluating the strength and durability of the sixty dentin bars. The minimum acceptable distance between the two supporting pins was 5 mm. The load was applied at the midpoint of the mounted dentin bar by the loading head and shaft, with a cross-head speed of 0.5 mm/min. Data was recorded on graph paper using a plotter to generate load-displacement curves. The load at which the dentin bars fractured was directly measured from the UTM software and cross-checked against the load-displacement curve for accuracy.

Measurement of flexural strength and modulus of elasticity

The load at the fracture site was used to determine FS. To calculate this value, the following equation was utilized (16): $FS = (3 * P * L) / (2 * b * d)$ The ME was calculated after drawing the slope of the tangent to the initial straight-line portion of the load deflection curve (16). Where ME is the modulus of elasticity in bending (Nm^2), we get; $ME = (L3\ m)/ (4 * b * d^2)$ where FS is the strength at the flexion (Flexural strength) (Nm^2), P is the load at the fracture moment (N), L is the span of support (m),B is equal to the tested dentin bar's width (m), d = the dentin bar's tested depth (m), m = the load-deflection curve's first straight-line portion's slope (Nm-1 of deflection). The value of ME is determined by calculating the slope of the linear section of the load-deflection curve.

Statistical analysis

The collected data from all groups were imported to Statistical Package for Social Sciences (SPSS, IBM SPSS version 20 Armonk, NY, USA). The standard descriptive methods such as mean, median, standard deviation, frequency, minimum and maximum were applied to determine the characteristics of the samples. Because the data did not meet the requirements for normality and homogeneity of variances assumptions, non- parametric One-Way Analysis of Variance (One-way ANOVA) was used to calculate the mean values for the FS and ME and multiple comparison between the experimental groups was determined by Tukey's B post hoc test. The confidence interval was set to 95 % and probability value $p < 0.05$ % was considered statistically significant.

Results

The mean values for FS were recorded as follows, from highest to lowest: Saline (168.38 MPa), CHD-FA (160.41 MPa), Maleic Acid (136.30 MPa), and EDTA (120.58 MPa). Similarly, the mean values for ME, from highest to lowest, were: Saline (11.15 GPa), CHD-FA (10.39 GPa), Maleic Acid (10.00 GPa), and EDTA (9.42 GPa). The highest FS and ME values were observed in the saline group (168.38 MPa and 11.15 GPa, respectively), while the lowest values (120.58 MPa and 9.42 GPa) were recorded in the EDTA group (Table 2).

According to the null hypothesis, it was assumed that the irrigants would not alter the FS and ME of dentin. However, since FS decreased with EDTA and Maleic Acid, and ME decreased with CHD-FA, EDTA, and Maleic Acid, the null hypothesis was rejected.

The results of the one-factor, independent measure ANOVA indicated significant differences ($p<0.001$) in both ME and FS among the four groups. To compare the mean values of the experimental groups, an independent sample t-test was performed. This test was used to determine whether there were significant differences between the mean values of two groups at a time. Pairwise comparisons for both ME and FS were conducted, and the results are summarized in Table 2.

When comparing the mean FS values, significant differences were observed between the following group pairs: EDTA vs. Maleic Acid, EDTA vs. CHD-FA, and EDTA vs. Saline, with the t-test indicating rejection of the null hypothesis. Table 1 clearly shows that EDTA had significantly lower FS values compared to Maleic Acid, CHD-FA, and Saline. Similarly, when Maleic Acid was compared to CHD-FA and Saline, it had significantly lower FS values than both. However, there was no significant difference in FS between CHD-FA and Saline.

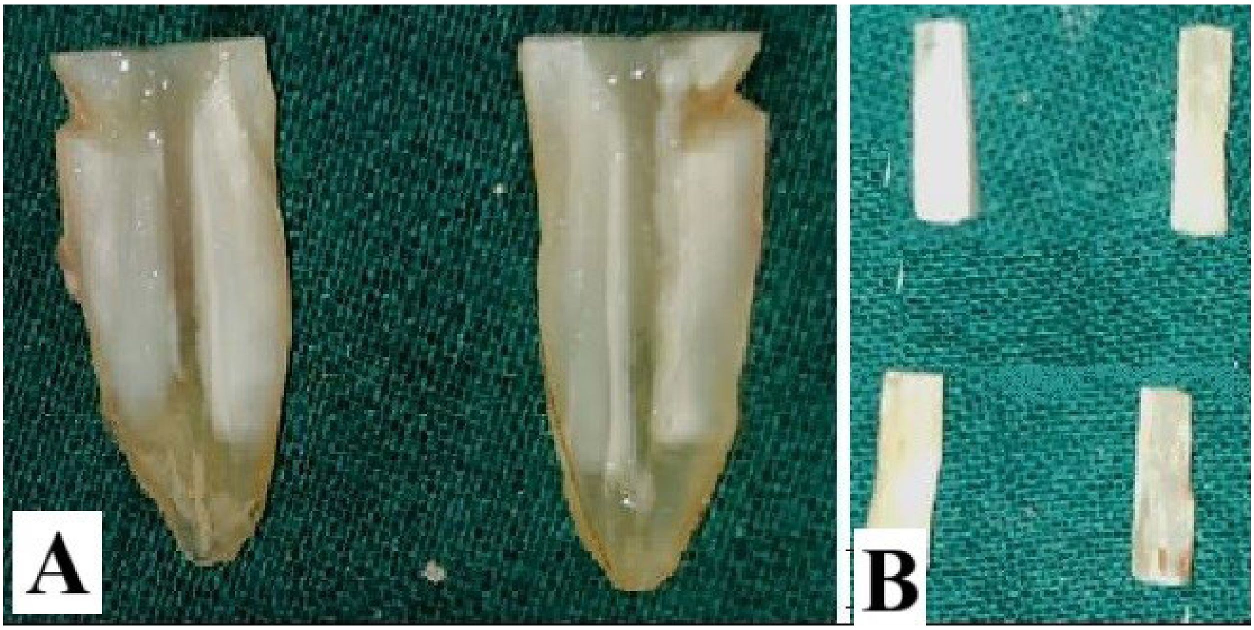

Figure 1. *Prepared tooth sample A) Longitudinal section of the prepared root; B) Four dentinal bars prepared.*

For ME, comparisons between EDTA vs. CHD-FA and EDTA vs. Saline revealed that EDTA had significantly lower values compared to both CHD-FA and Saline. Similarly, when CHD-FA was compared to Saline, and Maleic Acid was compared to Saline, the latter exhibited significantly higher values. However, the ME differences between EDTA and Maleic Acid, as well as between CHD-FA and Maleic Acid, were not statistically significant.

Overall, the results indicated that higher mean values were recorded for CHD-FA and Saline, whereas EDTA and Maleic Acid significantly reduced the FS and ME of radicular dentin. There was no significant difference in FS between CHD-FA and Saline. Similarly, for ME, no significant difference was observed between CHD-FA, Maleic Acid, and EDTA. However, the difference in ME between Saline and the other experimental groups was statistically significant.

Discussion

Endodontic therapy aims to achieve a root canal system free of microbiota and debris, which can then be sealed with obturating materials. Since 30% to 40% of the root canal remains untouched by mechanical instrumentation, irrigating solutions are used in conjunction to achieve complete disinfection (17). It has been proposed that chelating agents be used to remove smear layers, eliminate toxin-producing microorganisms from canal walls, and enhance the obturation seal (16). However, various irrigating solutions and chelating

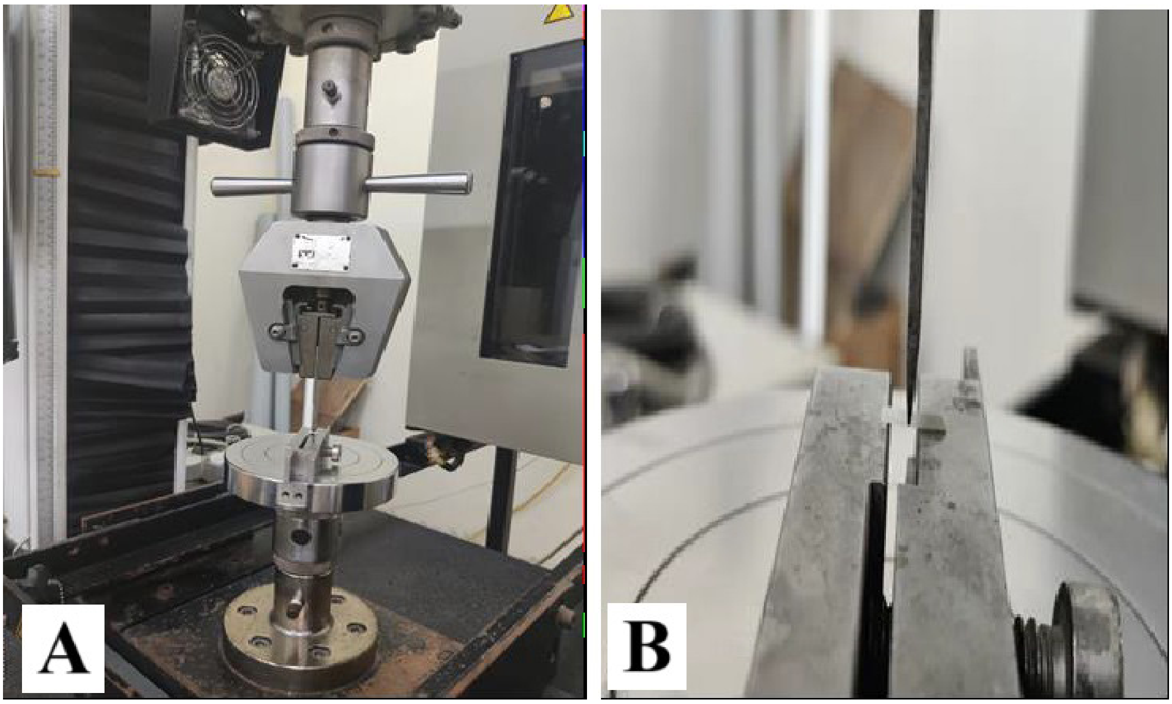

Figure 2. *A) Universal Testing machine B) Mounting of prepared sample for three-point flexural bending test.*

agents alter the mechanical properties and composition of radicular dentin, potentially compromising the fracture resistance of endodontically treated teeth (18). Chelating solutions soften dentin walls, facilitating effective smear layer removal and enabling the negotiation of calcified root canals. However, the degree of demineralization affects the physical and chemical characteristics of dentin. FS is influenced by flaws or alterations on the dentinal surface, and a decrease in FS indicates that less force is required for the cohesive bonds within dentin to fail (19). Therefore, it is preferable to use irrigants that do not negatively impact the mechanical properties of radicular dentin.

In this study, Smear Clear, a solution containing 17% EDTA with surfactants, was used. According to Dunavant *et al.* (20), after one and five minutes of contact time, Smear Clear eliminated 78.06% of biofilms. Smear Clear was used as the positive control group because various studies have demonstrated its superior efficacy compared to 17% EDTA in removing the smear layer from two-thirds of the root canal (21). Maleic acid (MA) used in this study exhibits dual properties, providing antibacterial action while also effectively removing the smear layer. MA has been shown to be superior to 17% EDTA in de-clogging the apical smear layer (10). A study suggested that MA could be beneficial in regeneration due to its low toxicity, chemical adherence to hydroxyapatite, and ability to support fibroblast attachment (22). Previous in vitro research has demonstrated that 7% maleic acid is more effective than 17% EDTA in removing the smear layer from the apical third of the root canal (21). A novel colloidal organic acid, CHD-FA, was included in this study due to its broad-spectrum antimicrobial and anti-inflammatory properties, as well as its ability to down-regulate infected cells. CHD-FA is a colloidal organic acid found in large amounts in humic acids. Humic substances, which are extracted from aquatic and soil sources, are known for their chelating properties with heavy metals, organic toxicants, and pesticides. CHD-FA can be derived from environmental sources or produced through the oxidation of coal or lignite (12). These substances effectively chelate heavy metals, inorganic anions, halogens, organic acids, and aromatic compounds (6). However, natural preparations often contain high levels of heavy metals and potentially toxic elements, making them unsuitable for human use. Recent advancements have enabled the development of CHD-FA through a wet oxidation process (23). A randomized controlled trial demonstrated that CHD-FA was well-tolerated in patients with eczema (24). CHD-FA, classified as a flavonoid (a plant metabolite), possesses antioxidant and wound-healing properties by activating cellular signalling pathways (25). It is therefore considered safe for use in treating conditions such as cancer, diabetes, neurological disorders, cartilage remodelling, and intestinal pathologies.

The objective of this study was to evaluate the impact of different root canal irrigants, including MA, CHD-FA, and EDTA, on the FS and ME of radicular dentin. Freshly prepared dentin bars were obtained from radicular dentin with standardized dimensions of 7 mm in length, 1 mm in width, and 1 mm in height, following the protocols of various studies (18,20). The dentin bars were stored in 100% humidity containing 0.1% chloramine T to prevent dehydration before being subjected to a three-point bending test. A study by Huang *et al.* (1992) demonstrated that dehydration decreases flexibility, increases brittleness, and reduces the elastic modulus in tooth samples with no pulp (26).

Previous research by Dua *et al.* (21) and Patterson *et al.* (27) revealed that a one-minute exposure was insufficient for cleaning the apical third, whereas a five-minute contact time was effective for intra-canal smear layer removal in clinical practice. Consequently, the present study maintained a uniform immersion period of five minutes for all experimental groups.

In a typical clinical setting, root canals are obturated. However, obturation was not performed in this study, as the primary objective was to evaluate the effects of irrigants on the FS and ME of radicular dentin (28). The three-point bending test is a standardized method for assessing material flexibility and elasticity. Dentin strength and durability can be evaluated using parameters such as flexural strength, ultimate tensile strength, and fracture resistance. To determine the effects of chemical agents on healthy dentin, a three-point bending test was performed using a UTM, with a crosshead speed of 0.5 mm/min to ensure accurate load-displacement curve recordings (29).

The results demonstrated that CHD-FA and saline groups exhibited higher mean FS values compared to MA and EDTA. In terms of ME, saline showed statistically higher mean values than the other experimental groups. The higher FS and ME values observed in the CHD-FA group may be attributed to its relatively high pH (3.6) compared to MA (1.7), which does not adversely affect FS and ME values. This finding aligns with a study by Hiradate *et al.* (30), which stated that CHD-FA reduces demineralization and dehydration. CHD-FA contains a high concentration of polyphenolic compounds with strong antioxidant activity, acting as effective free radical scavengers. These compounds neutralize hydroxyl radicals, peroxynitrite,

Table 2. *Means and pair-wise comparisons for flexural strength and modulus of elasticity among study groups*

			Pair-wise comparison for FS and ME (P Value)							
			Saline		Maleic acid		CHD-FA		EDTA	
Group	**FS (MPa) Mean ± SD**	**ME (GPa) Mean ± SD**	**FS**	**ME**	**FS**	**ME**	**FS**	**ME**	**FS**	**ME**
Saline	168.38±26.90	11.15 ±0.85	-	-	.001*	.001*	.422	.023*	.000*	.000*
Maleic acid	136.30±15.09	10.00 ±0.92	.001*	.001*	-	-	.006*	.248	.027*	.116
CHD-FA	160.41±26.65	10.39±0.90	.422	.023*	.006*	.248	-	-	.000*	.011*
EDTA	120.58±21.30	9.42±1.04	.000*	.000*	.027*	.116	.000*	.011*	-	-

FS: Flexural strength; ME: Modulus of elasticity; SD: Standard deviation; p: Probability value; *Indicates significant difference between the groups.

hydrogen peroxide, hypochlorous acid, superoxide, and singlet oxygen (14). As an antioxidant, CHD-FA reverses the oxidative effects of residual NaOCl, commonly used as an irrigant, thereby restoring the redox potential of oxidized dentin. It binds to superoxide radicals and recycles other antioxidants by removing free radicals (31). CHD-FA, classified as a flavonoid within the polyphenol group, interacts with proline-rich proteins, which have a high affinity for flavonoids. Proline, a key component of dentin collagen, forms hydrogen bonds with the hydroxyl groups of flavonoid molecules (25). This mechanism facilitates flavonoid-induced cross-linking of protein molecules, preserving the biomechanical integrity of the collagen matrix (32). This phenomenon may explain the higher FS and ME observed in the CHD-FA group. Similar findings were reported by Epasinghe *et al.* (33), who demonstrated that flavonoid treatments improved the ME and ultimate tensile strength of demineralized dentin.

In this study, MA exhibited the second lowest mean FS and ME values, followed by EDTA, indicating a reduction in the mechanical properties of radicular dentin. The acidity of MA has been proposed to contribute to dentin erosion and decreased microhardness. The demineralization of root canal dentin may have resulted from the low pH (1.7) of the MA solution, potentially leading to adverse effects. MA alters dentin surface morphology, increasing surface roughness and compromising mechanical properties. Its demineralizing effect negatively impacts both peritubular and intertubular dentin. Matos *et al.* (34) concluded that MA alters dentin morphology by reducing collagen content, which may further impair mechanical properties. MA decalcifies root dentin, removing significant amounts of calcium and phosphorus within the first five minutes. Ballal NV et al. (22) found no significant difference in the microhardness of radicular dentin after five minutes of exposure to EDTA and MA, which aligns with the findings of the present study.

Smear Clear (17% EDTA with cetrimide and two surfactants: polyoxyethylene and iso-octylcyclohexyl) exhibited the lowest mean FS and ME values compared to the other experimental groups. This finding is supported by studies such as Durmus *et al*. (35), which reported that the lowest elastic modulus values were observed when Smear Clear was used in combination with NaOCl on root dentin. The addition of surfactants to EDTA has been suggested to induce structural changes and reduce microhardness, although no significant difference in microhardness was observed between EDTA alone and EDTA combined with cetrimide (36). Several studies have reported that EDTA alters the chemical structure of human dentin and modifies the calcium-to-phosphorus (Ca/P) ratio on the dentin surface (10,27). By removing Ca^{2+} ions from root dentin, EDTA influences dentin's mechanical properties. Changes in the Ca/P ratio may alter the initial organic-to-inorganic component ratio, potentially affecting dentin permeability and solubility. Strong demineralizing effects, resulting in dentinal tubule enlargement, dentin softening, and collagen denaturation, likely contribute to the reduction in dentin FS (37).

This study provides valuable insights into the efficacy of CHD-FA as an alternative to conventional irrigants for root canal treatment. The findings suggest that CHD-FA effectively removes the smear layer while preserving the mechanical properties of root canal dentin.

Conclusion

This study confirmed that CHD-FA did not deteriorate the mechanical properties of radicular dentin and can be considered an effective alternative irrigant in endodontic therapy. In contrast, EDTA and maleic acid were found to decrease the flexural strength (FS) and modulus of elasticity (ME) of radicular dentin.

Türkçe öz: *Maleik Asit, Karbonhidrat Türevi Fulvik Asit ve Etilendiamin Tetra Asetik Asidin İnsan Radiküler Dentininin Bükülme Direnci ve Elastisite Modülü Üzerindeki Etkisi: İn Vitro Bir Çalışma. Amaç: Bu çalışma, etilendiamin tetra-asetik asit (EDTA), karbonhidrat kaynaklı fulvik asit (CHD-FA) ve maleik asidin (MA) insan radiküler dentininin bükülme direnci (FS) ve elastisite modülü (ME) üzerindeki etkisini değerlendirmeyi amaçlamaktadır. Gereç ve Yöntemler: Tek köklü ve tek kanallı on beş adet taze çekilmiş insan mandibular premolar dişi seçildi. Dişler, sement mine birleşiminin (CEJ) 2 mm altından dekape edildi ve kökler bukkal ve lingual yarılara ayrıldı. Her bir yarıdan, standartlaştırılmış iki düzlemsel paralel dentin çubuğu (7 mm uzunluk × 1 mm genişlik × 1 mm yükseklik) hazırlandı. Örnekler dört gruba ayrılarak 5 dakika boyunca ilgili irrigasyon solüsyonlarına daldırıldı: serum fizyolojik (kontrol), maleik asit, CHD-FA ve EDTA. Daha sonra, FS ve ME üç noktalı eğme testi kullanılarak değerlendirildi. Bulgular:* *Hem EDTA hem de maleik asit, radiküler dentinin FS ve ME değerlerini azalttı. Ancak, çalışma CHD-FA ve serum fizyolojik (kontrol) gruplarında, EDTA ve maleik asit gruplarına kıyasla daha yüksek ortalama FS ve ME değerlerinin kaydedildiğini göstermiştir. Sonuç: CHD-FA, radiküler dentinin mekanik özelliklerini, özellikle FS ve ME'yi olumsuz etkilemediğinden, endodontik irrigasyon solüsyonu olarak önerilmektedir. Anahtar Kelimeler: radiküler dentin; bükülme direnci; elastisite modülü; EDTA; CHD-FA; maleik asit.*

Ethics Committee Approval: The protocol of this in vitro study was approved by the Institutional Ethical Committee of Sree Balaji Dental College & Hospital, Bharath Institute of Higher Education and Research (Approval number: SBDCH/IEC/12/2020/25).

Informed Consent: Participants provided informed consent.

Peer-review: Externally peer-reviewed.

Author contributions: Author contributions: LS, VP, SM participated in designing the study. LS, VP, SM participated in generating the data for the study. LS, VP, SM, RP, AV, KM, PEM participated in gathering the data for the study. LS, VP, SM, RP, AV, KM, PEM participated in the analysis of the data. LS, VP, SM wrote the majority of the original draft of the paper. LS, VP, SM, RP, AV, KM, PEM participated in writing the paper. LS, VP, SM, RP, AV, KM, PEM have had access to all of the raw data of the study. LS, VP, SM, RP, AV, KM, PEM have reviewed the pertinent raw data on which the results and conclusions of this study are based. LS, VP, SM, RP, AV, KM, PEM have approved the final version of this paper. LS, VP, SM, RP, AV, KM, PEM guarantee that all individuals who meet the Journal's authorship criteria are included as authors of this paper. AT, BS, ED, VS guarantees that all individuals who meet the Journal's authorship criteria are included as authors of this paper.

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