TO COMPARE & EVALUATE THE EFFECT OF VARIOUS CHELATING AGENTS ON CALCIUM CONTENT AND MICROHARDNESS OF ROOT DENTIN- AN IN VITRO STUDY

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LIST OF ABBREVIATIONS

Sr. no	Abbreviations	Full form
01.	s	Seconds
02.	mm	Millimeter
03.	ml	Milliliter
04.	RVG	Radiovisiography
05.	$Ca(OH)_2$	Calcium Hydroxide
06.	° C	Degrees Celsius
07.	h	Hours
08.	min	Minutes
09.	CEJ	Cemento-Enamel Junction
10.	SEM	Scanning Electron Microscopy
11.	OSHA	Occupational Safety and Health Administration
12.	CDC	Centre for Disease Control
13.	ANOVA	Analysis of Variance
14.	BMDP	Biomedical Data Processing
15.	SD	Standard Deviation
16.	S	Significant
17.	NS	Not Significant
18.	HS	Highly Significant
19.	n	Number of specimens
20.	p-value	Probability of obtaining a test statistic at least as extreme as the one that was actually observed
21.	°	Degrees
22.	Max.	Maximum
23.	Min.	Minimum

Sr. no	Abbreviations	Full form
24.	No.	Number
25.	NaOCl	Sodium Hypochlorite
26.	EDTA	Ethylene Diamine Tetra-acetic Acid
27.	$EDTAHNa_3$	Sodium Ethylene Diamine Tetra-acetic Acid
28.	CI	Confidence Interval
29.	PDL	Periodontal Ligament
30.	etc	et cetera
31.	µm	Microns
32.	mins	Minutes
33.	Ca	Calcium
34.	Ca^{2+}	Calcium Ion
35.	Ca/P	Calcium/Phosphorus
36.	mol L^{-1}	Moles per liter
37.	CHX	Chlorhexidine
38.	ppm	parts per million
39.	VHN	Vickers hardness Number

INTRODUCTION

INTRODUCTION

What we remove from the pulp space,

Is far more important than what we replace it with.

The success of endodontic treatment is mainly dependent on thorough cleaning, shaping and disinfection of the root canal system through the use of instruments and effective irrigant solutions.[1]

Biomechanical preparation when done using manual or rotary instruments leads to accumulation of smear layer, which is composed of organic and inorganic particles of calcified tissues, necrotic tissue, odontoblastic processes and microorganisms. This layer of approximately 1 to 2 μm in thickness that covers the

prepared canal walls and occludes the orifices of the dentinal tubules. This hinders the penetration of intracanal medicaments and sealers into the dentinal tubules.[2]

Removal of the smear layer improves the fluid tight seal of the root canal system. It is believed that removing this layer could dissolve attached microbiota and their toxins from root canal walls, improve the seal of root fillings, and reduce the potential of bacterial survival and reproduction.[3]

Irrigating protocols play a key role in removing smear layer and cleaning the area that cannot be directly reached by instrumentation during endodontic treatment.[1] Sodium hypochlorite removes only the organic component of smear layer whereas inorganic component demands the use of chelating agents like EDTA, Citric acid, MTAD, Etidronate, Chitosan, etc.

EDTA is a sodium salt of ethylene diaminetetraacetic acid and the most popular chelating agent used during endodontic therapy.[4] It has the ability to chelate with metal ions in a 1:1 metalo-EDTA complex. It is generally used to remove inorganic debris and smear layer from the root canal.[5] Demiray Kökçü et al. (2016) concluded that 5% and 17% EDTA were equally effective in removing debris and smear layer.[6]

Another chelating agent that is used in the canal for smear layer removal is citric acid. It is a weak organic acid used at 5-50% concentration. It is found in tissue and thus does not have cytotoxic effect as EDTA. It has antimicrobial properties.[7] Goldman et al reported that the effect on the smear layer removal obtained with citric acid were similar to those with EDTA.[8] Various other study reported that it removes

smear layers better than many acids such as polyacrylic acid, lactic acid, and phosphoric acid.[1]

Chitosan is a natural polysaccharide obtained by the de-acetylation of chitin, which is found in crab and shrimp shells. It has a high chelating ability for various metal ions in acidic conditions and has been applied widely for the removal or recovery of metal ions in different industrial areas. It has attracted attention in dental research because of its biocompatibility, biodegradability and lack of toxicity.[9] Silva P.V et al found that 17% EDTA, 0.2% chitosan and 15% citric acid had similar smear layer removal capacity.[10]

Depending on concentration, duration of exposure, and other factors, chelators can demonstrate a proteolytic action by hydrolysis of long peptide chains such as collagen and reacts with the calcium ions in the hydroxyapatite crystals which can cause changes in the microstructure of the dentin by changing in the Ca/P ratio. These may lead to alterations in mineral content of dentin. Cobankara et al reported that there was significant loss of calcium ions with Peracetic acid, Citric acid, and EDTA.[11]

Microhardness is an important property that determines the long term prognosis of endodontically treated teeth. Any change in the Ca/P ratio may alter the original proportion of organic and inorganic components, which in turn affects the microhardness, permeability, and solubility characteristics of dentin and alters the adhesion of dental materials such as resin-based sealers and cements to dentin.[12] De-Deus G et al found that microhardness of dentin decreased with increasing time of

application of chelating agent.[13]Therefore, the effect of chelating agents on Calcium depletion and its effect on microhardness of root dentin needs to be evaluated.

Different methods such as atomic absorption spectrometry, Flame photometry, complexometric titration with EDTA, SEM, Energy disperasive spectrometer, Fourier Transform Infrared (FTIR) or Inductively Coupled Plasma–Atomic Emission Spectrometry (ICP-AES) are used to evaluate the demineralization effect of different chemicals, provided that calibration is accomplished precisely. The advantage of ICP-AES technique is that the mineral content of dentin can be measured at concentrations of parts per billion (g/L) and multiple elements can be measured at the same time.[11]

Microhardness is sensitive to composition and surface changes of the tooth structure. It has been indicated that microhardness determination can provide indirect evidence of mineral loss or gain in dental hard tissues.[14] Vickers indenter microhardness test is the most commonly used method to measure the hardness of dentin.[15]

Due to lack of available documentation, this study was undertaken to evaluate & compare the effect of various chelating agents, used during irrigation in endodontic procedures, in removal of calcium ion and its effect on microhardness of root dentin.

Therefore, the null hypothesis proposed was that there is no difference between EDTA, Citric Acid and Chitosan in their ability to remove calcium ions and their effect on microhardness of root dentin.

AIM AND OBJECTIVES

AIM & OBJECTIVES

AIM:

"To compare & evaluate the effect of various Chelating Agents on calcium Content and microhardness of root dentin"

OBJECTIVES:

1) To evaluate the effect of EDTA, Citric acid and Chitosan on calcium content of root dentin.

2) To evaluate the effect of EDTA, Citric acid and Chitosan on microhardness of root dentin.

3) To compare the effect of EDTA, Citric acid and Chitosan on calcium loss of root dentin.

4) To compare the effect of EDTA, Citric acid and Chitosan on microhardness of root dentin.

5) To compare the effect EDTA, Citric acid and chitosan on calcium content and microhardness of root dentin.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The current methodologies of smear layer removal from the canals during endodontic therapy, require the use of chelating agents alone or in combination. Chelating agents also aid in the preparation of narrow and calcified root canals during endodontic therapy by softening dentin. Therefore, it is essential to critically examine various researchers studying the effect of chelating agents on dentin.

Nygaard-Ostby (1957)[3] analysed the extent of demineralization by 15% EDTA at different time intervals using polarized microscope. They measured demineralization zone encircling around the root canal lumen and found that extension of demineralization was time dependant (20mins – 96hrs). A 20- 30µm of demineralized zone was apparent after 5 minutes. Zone of 30-40µm after 30 minutes

and 50µm after working time of 24-48hrs. They also found that EDTAC had a rapid demineralization effect on comparison to EDTA, as it had a lower surface tension and concluded that EDTA has self limitating action.

Seidberg and Schilder (1974)[16] using gravimetrical analysis, showed that the property of EDTA was self limitating. This limitation is thought to be because of changes in pH during demineralization of dentin. Under neutral condition, most chelators have a pH near neutral value, 99% of the EDTA is present as EDTAHNa3. The exchange of calcium from the dentin by hydrogen results in a subsequent decrease in pH. Because of release of acid, the efficiency of EDTA decreases with time, on the other hand, the reaction of the acid with hydroxyapatite affects the solubility of dentin.

Cergneux M et al (1987)[2] studied the influence of smear layer on sealing ability of canal obturation cleaned either chemically with EDTA or mechanically with ultrasonic technique. He examined 60 single rooted teeth for the amount of smear layer using scanning electron microscope, all specimens were then subjected to dye infiltration before being transversely sectioned at various levels. The amount of leakage was scored on an arbitrary four-point scale.

In the control group, under scanning electron microscope, canals walls were covered with smear layer. This contrasted in other two groups which showed little or no smear layer. Also, there was some difference in leakage between the groups at a different level. EDTA treated canals showed the least infiltration, while those treated with ultrasound showed significantly less leakage as compared with the control group.

Hennequin M et al (1994)[17] evaluated the effect of different pH values (0.8, 1.1, 1.3, 1.5 and 1.7) of citric acid solutions on the calcium and phosphorus content at the cervical, middle and apical third of eighteen mandibular incisors, using microprobe analyser. It was concluded that there was no significant difference in the loss of calcium values between the three radicular levels. The citric acid solution at pH 1.1 produced more calcium loss as compared to all other solution. A scanning electron microscopic examination of dentin surface showed that the decreasing pH of citric acid solutions resulted in progressive disappearance of smear layer and was totally eliminated at pH <1.5.

Hennequin M et al (1995)[18] determined the effects on the Ca, P and Mg contents of human dental roots before and after a citric acid treatment (pH=l) on 25 human mandibular third molars, using an electron microprobe analyser associated with a scanning electron microscope. The measurements were made on transverse sections through the cervical 1/3 of the molar roots. The measurements were performed at the following 8 levels: the internal cementum, the cementum-dentin junction, cementum-related dentin, 4 external dentin levels located at 220 µm, 420 µm, 620 µm and 820 µm from the cementum- dentin junction, and finally the juxta-pulpal dentin.

Results showed that there was a loss in Ca and P levels but not in Mg. Also, an acid-resistant dentin layer of 600 µm was found under the cementum-dentin junction which showed an increase in the Ca/P ratio in this layer.

Yamaguchi M et al (1996)[19] investigated the effect of citric acid (0.1M, 0.5M, 1M, 2M) and EDTA (0.5M) solutions as decalcifying and cleansing agents in

root canal irrigation using 3:1 powdered dentin-resin mixtures at 5, 10, 30, 60 and 120 minutes and their antibacterial effect using twelve bacterial strains (Staphylococcus aureus, Streptococcus sanguis, Lactobacillus casei, Actinomyces naeslundii, Peptococcus niger, Peptostreptococcus anaerobius, Bifodobacterium Bifidum, Eubacterium lentum, Propionibacterium avidum, Veillonella parvula, Porphyromonas endodontalis, Fusobacterium nucleatum) isolated from infected root canals.

Results showed that the 0.1M citric acid extracted least amount of calcium. The amount of calcium extracted by 0.5, 1 and 2M citric acid solution was more than that of 0.5M EDTA at 60 and 120 minutes. Citric acid solution of 1 and 2M concentrations showed strong antimicrobial action against A. naeslundii, P. anaerobius, B. bifidum, E. lentum, P. avidum, V parvula and F. nucleatum. Antimicrobial action of EDTA was similar to the action of 1 and 2M citric acid.

Di Lenarda et al (2000)[20] studied effectiveness of 1 mol L^{-1} Citric acid and 15% EDTA irrigation on smear layer removal using scanning electron microscope. Eighty-one single-rooted human teeth were divided on the basis of irrigation protocol: 5% NaOCl alone, NaOCl alternated with 1 mol L^{-1} citric acid solution and a combination of 15% EDTA and Cetrimide solution. After longitudinal sectioning, dentinal walls were micro-photographed with scanning electron microscopy at 300x and 1000x magnifications. Qualitative and quantitative cleansing level evaluations were performed.

In qualitative evaluation, the ability NaOCl in removing debris from dentinal wall at 1000x magnification confirmed a clear lack of effect in every area. In the apical third the best results were obtained using the citric acid solution. In quantitative

evaluation, within the different instrumentation methods used, the EDTA and citric acid irrigants gave significantly better results than NaOCl ($P < 0.001$). Within the group treated with manual instrumentation, EDTA produced significantly cleaner walls.

Dogan H and Calt S (2001)[21] evaluated the effects of combined and single use of EDTA, RCPrep, and NaOCl on mineral content of root dentin using scanning electron microscopy and energy dispersion spectrometric microanalysis. Thirty-six standardized midroot dentin specimens obtained from human anterior teeth were used to determine the calcium, phosphorus, and magnesium levels after treatment.

The results showed that EDTA combined with NaOCl irrigation as final flush and NaOCl alone changed the calcium/phosphorus ratio of root dentin significantly; and there was a significant increase in the magnesium level after the use of chelating agent combined with NaOCl.

Hulsmann M et al (2002)[22] compared the effect of three (paste-type) chelating agents (Calcinase-Slide, Glyde-File & RCPrep) on the root dentin. Changes in microhardness and loss of weight was calculated after 3, 6 and 9 minutes. It was found that loss of hardness increased significantly with the increased time of contact of the chelating pastes with dentin. There were significant differences between the three chelating agents and the control for loss of weight. After 3 min there was no significant differences between the chelating agents, after 6 and 9 min Calcinase-Slide showed significantly more weight loss than RC-Prep; after 6 min Glyde-File showed significantly more weight loss than RC-Prep.

Slutzky-Goldberg I et al (2004)[23] designed a study to evaluate the effect of 2.5% and 6% sodium hypochlorite for 5, 10, or 20 min and at 500 µm, 1000 µm, and 1500 µm depths on root dentin microhardness on forty-two bovine roots and concluded that decrease in microhardness was found at 500 µm with samples irrigated with 6% and 2.5% NaOCl at all irrigation periods. There also was a significant difference in groups irrigated for 10 and 20 min where the decrease in microhardness was more marked after irrigation with 6% NaOCl than 2.5% NaOCl.

Scelza MF et al (2004)[24] studied the effect of EDTA-T, 17% EDTA, and 10% citric acid on the removal of smear layer from root canal dentin after final irrigation for 3, 10, and 15 min on 90 extracted human canines. Results showed that irrigation with 10% citric acid for 3 min was significantly better in removing smear layer when compared to 10 and 15 min results, and when irrigation with EDTA for 3 min was compared to 15 min. In all cases, irrigation for 3 min presented the greatest number of open dentinal tubules. There were no significant differences among the 3 time intervals of irrigation for EDTA-T, although there were greater numbers of open tubules at 3 mins.

Machado-Silveiro LF et al (2004)[25] measured the demineralization capability of 1 and 10% citric acid, 10% sodium citrate and 17% EDTA during immersions of 5, 10 and 15 min on root canal dentin. Crowns were sectioned from eight maxillary canines. The cementum was removed and a 3-mm thick cross-sectional slice was obtained from the cervical third of each root. Each slice was sectioned into four equal parts. These specimens were assigned to one of four groups (n = 8) for the application of 1% citric acid, 10% citric acid, 10% sodium citrate or 17% EDTA. Each specimen

underwent three successive 5-min immersions in each solution at room temperature. Two millilitres of solution were collected from the extracts and lanthanum oxide was added for the calcium reading by ICP- AES technique.

They concluded that 1 and 10% citric acid were more effective than EDTA or sodium citrate at the three immersion times. 10% citric acid was more effective than 1% citric acid. EDTA and 1 and 10% citric acid showed decreasing effectiveness with time, and the decrease was significant for citric acid at both concentrations. Although sodium citrate removed little calcium during the three time periods, the small increase recorded was significant.

Nakashima K et al (2005)[26] evaluated the influence of smear layer removal with 3% & 15% EDTA solution (pH of 9.0) in terms of the permeability of root canal disinfectants into the dentin, wetting by endodontic sealer, and adhesive strength of the sealer**.**

They concluded that no inhibition zones were observed for any disinfectant in the untreated group, whereas inhibition zones were observed with both 3% & 15% EDTA groups.

The contact angle formed by sealer was lower in the 3% EDTA group than in the 15% EDTA group for Canals, Canals N, Apatite Root Sealer and AH26. The contact angle was higher in each EDTA group than in the untreated group. Also, Adhesive strength of each type of sealer to dentin was higher in the EDTA groups than in the untreated group.

Ayce Unverdi Eldeniz et al (2005)[27] investigated the effect of citric acid and EDTA solutions on the microhardness and the roughness of human root canal dentin. Forty five Specimens were randomly divided into three groups and were treated as follows: (a) one molar (19%) citric acid (C6H8O7) for 150 s followed by 5.25% NaOCl; (b) 17% EDTA for 150 s and rinsed with 5.25% NaOCl; (c) rinsed with distilled water and served as control. Three groups were then divided into two subgroups. First subgroup were subjected to Vicker's testing whereas the second subgroup underwent surface roughness testing. It was found that there was Significant differences were observed in microhardness with EDTA and citric acid the increase in dentin roughness after citric acid treatment was significantly greater than the EDTA treated.

Teixeira CS et al (2005)[28] studied the influence of irrigation time with ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite (NaOCl) on intracanal smear layer removal under the scanning electron microscope. They selected twenty-one extracted human permanent teeth with single straight root canals. The root canals of the teeth were instrumented and, at the end of preparation, were irrigated with 3 mL of 15% EDTA, followed by 3 mL of 1% NaOCl for 1 min (group 1), for 3 min (group 2), and for 5 min (group 3). The canals of teeth in group 4 (control) did not receive the final irrigation. The teeth were sectioned longitudinally and prepared for an SEM. The dentinal wall of cervical, middle and apical thirds was graded according to the amount of debris and smear layer remaining on the walls.

It was reported that in Group 1 the smear layer on the dentin wall of the cervical and middle thirds was removed completely. The entrances to the dentinal

tubules were visible and slightly enlarged. On the apical third, the dentin smear layer was partly removed on five of the six analysed specimens. When compared with the other thirds in this group, a significant difference was seen. While in Groups 2 (3 min) and 3 (5 min) results for these two groups were identical. The dentin wall of the cervical and middle thirds on all the samples were free from smear layer, the entrances to the tubules were visible and enlarged. Despite no significant difference being seen between the thirds, smear layer was completely removed from the apical third on four specimens, and in two of them the dentin surface was partly covered as in group 1. Overall, comparing the thirds between experimental groups 1, 2 and 3, no significant difference was found.

Zehnder et al (2005)[29] assessed the interactions of EDTA and citric acid (CA) with sodium hypochlorite (NaOCl) for available chlorine (HOCl OCl–) contents in these mixtures using a standard iodine/thiosulfate titration method followed by calcium chelation and smear layer removal ability using Atomic absorbtion spectrometry and SEM evaluation respectively of these solution in sixty single rooted premolars.

They reported that EDTA and Citric acid solution almost completely loosed OCl-/HOCl immediately upon mixing with NaOCl. Also, there was a tendency for Citric Acid to remove more smear layer than EDTA. The presence of NaOCl in mixtures had no impact on calcium complexing agents with respect to their calcium chelating capacity or smear layer removal. There was a strong negative correlation between calcium concentrations in eluates and amounts of smear layer remaining in root canals.

De-Deus G et al (2006)[13] evaluated the effect of citric acid, ethylenediaminetetraacetic acid (EDTA) and ethylenediaminetetraacetic acid plus Cetavlon (EDTAC) solutions on the microhardness of sixteen maxillary human canines. Their results indicated that microhardness decreased with increasing time of application of chelating solutions. There were no significant ($P > 0.05$) differences between initial microhardness for the three groups as well as after 1 min of application of the substances. After 3 min, EDTA produced a significantly greater reduction in microhardness. However, there was no significant difference between EDTA and EDTAC after 5 min. Citric acid caused significantly less reduction in microhardness. Overall, citric acid was least effective in reducing dentin hardness whilst EDTA had the strongest effect.

Lui et al (2007)[30] assessed the in vitro efficacy of Smear Clear (Sybron Endo, Orange, CA, USA), a 17% EDTA solution with surfactants, and 17% EDTA with and without the use of ultrasonics in removal of the smear layer. Seventy-five extracted teeth were randomly distributed into five test groups, the root canals were prepared by using ProFile rotary NiTiinstruments (Dentsply Maillefer, Ballaigues, Switzerland) and subjected to different final irrigating regimes. Samples were examined under the scanning electron microscope and scored for debris and smear layer removal. Statistical analysis showed that groups containing Smear Clear and Smear Clear with ultrasonic activation did not perform significantly better than groups containing 17% EDTA and 17% EDTA with ultrasonics. Addition of surfactants to EDTA in Smear Clear did not result in better smear layer removal, although, the use of ultrasonics with 17% EDTA improved the smear layer removal.

Sayin et al (2007)[31] carried out a study to determine the extent of calcium removal on root canal dentin after 17% EDTA, 17% EGTA, 15% EDTAC, and 1% tetracycline-HCl treatment; with or without subsequent use of 2.5% NaOCl. The samples were immersed in the test solutions for 1 and 5 minutes, after which the amount of calcium ion (Ca2_) release into the solutions was determined by flame photometry.

Regardless of treatment time, all single (treatment solution only) and combined (treatment solution with subsequent NaOCl application) irrigation regimens removed significantly more Ca^{+2} than control treatment. Treatment with 17% EDTA and 17% EDTA + 2.5% NaOCl resulted in the maximum amount of Ca^{+2} removal from root canal dentin. For 1-minute treatment, there was no significant difference between the Ca^{+2} release values of tetracycline-HCl and 2.5% NaOCl. Within each test group, extending the treatment time to 5 minutes resulted in significantly more Calcium removal.

Thangaraj D et al (2009)[32] determined the calcium loss and its effect on Microhardness of the root canal dentin following treatment with aqueous solution of 17% EDTA at different time intervals (1, 2, 3, 4, 5, 6 and 7 minutes). Evaluation of amount of calcium release into the solution by Atomic Absorption Spectrophotometer and microhardness was tested using a microhardness testing machine.

It was concluded that 17 % EDTA solution resulted in the maximum amount of calcium loss as the treatment time increased, and also there was a decrease in the dentin microhardness as the treatment time with 17% EDTA solution was increased.

Spano et al (2009)[33] evaluated the concentration of calcium ions and smear layer removal ability of 15% ethylenediaminetetraacetic acid (EDTA), 10% citric acid, 10% sodium citrate, apple vinegar, 5% acetic acid, 5% malic acid, and sodium hypochlorite in forty-two human maxillary central incisors when kept in contact for 5 minutes. The concentration of calcium ions was measured by using flame atomic absorption spectrometry, and smear layer removal was determined by scanning electron microscopy.

The results concluded that the solutions containing 15% EDTA and 10% citric acid had removed greater concentrations of calcium ions than apple vinegar, 5% acetic acid, 5% malic acid, and 10% sodium citrate. Also, 15% EDTA and 10% citric acid were the most efficient irrigants in removing smear layer.

Scelza MF et al (2010)[34] evaluated the inflammatory response of 17% EDTA, 17% EDTA-T, and 10% citric acid in bony defect created in rat jaws at 1st, 7th, 14th, and 28th day. Hemijaws were prepared for light microscopy, and samples were stained with haematoxylin and eosin. It was concluded that for all days, 10% citric acid and 17% EDTA-T showed, respectively, the lowest and highest number of inflammatory cells per area. All tested substances and controls showed the highest inflammatory cell response on the 14th day.

Cobankara FK et al (2011)[11] in an in vitro study assessed the effect of several chelating agents (17% EDTA, 10% citric acid, 18% etidronate, 2.25% peracetic acid on the various mineral [Calcium(Ca), Phosphorus (P), Potassium(K), Manganese(Mn), Sodium(Na), Zinc(Zn), and Sulphur(S)] content of root dentin.

It was concluded that peracetic acid significantly decreased P, K, Mg, Na, and S levels of root dentin compared with the other groups. The S level of root dentin decreased by different levels in all chelating solutions compared with the control group and the greatest decrease was observed in the peracetic acid group. There were significant decreases in the Ca levels of root dentin after treatment with peracetic acid, citric acid, and EDTA compared with the other groups. The Mn level of root dentin significantly decreased in the citric acid and peracetic acid groups. Na and Zn levels significantly decreased in the peracetic acid, citric acid and etidronate groups. However, peracetic acid had a tendency to show a lower Ca/P ratio than the other solutions, and EDTA, etidronate, and citric acid had a tendency to show a higher Ca/P ratio.

Cruz-Filho et al (2011)[15] evaluated the effect of different chelating solutions (15% EDTA, 10% citric acid, 5% malic acid, 5% acetic acid, apple vinegar, 10% sodium citrate) on the microhardness of the most superficial dentin layer from the root canal lumen. Results showed that EDTA and citric acid had the greatest overall effect, causing a sharp decrease in dentin microhardness without a significant difference from each other. Apple vinegar, acetic acid, and malic acid were similar to each other and presented intermediate results. Sodium citrate and deionized water were similar to each other and did not affect dentin microhardness.

Mishra L et al (2012)[35] assessed calcium loss from root canal dentin following EDTA and Tetracycline – HCL Treatment with or without subsequent NaOCl Irrigation and evaluated their effect on microhardness of dentin. The one half of the specimen is used for the Calcium loss evaluation by ICP-AES technique and

the other one half with the acrylic block is used for the micro hardness study. Results showed that in a group of 17% EDTA + 2.5% NaOCl resulted in maximum Calcium loss and reduction in the microhardness. But in Group 1%Tetracycline HCl + 2.5% NaOCl solution effectively removed Calcium without much altering the microhardness of the root dentin.

Pimenta J. A et al (2012)[36] studied the effect of 0.2% chitosan, 15% EDTA and 10% citric acid on the microhardness of root dentin using Knoop hardness microhardness tester and then examined by scanning electron microscopy. The groups treated 0.2% chitosan, 15% EDTA and 10% citric acid reduced dentin microhardness in a statistically similar manner to each other, but significantly different from the control. SEM micrographs revealed that all chelating solutions removed smear layer from the middle third of the root canals, leaving visible, open dentinal tubules.

Silva PV et al (2013)[10] evaluated the efficacy of smear layer removal using chitosan compared with different chelating agents, and quantified the concentration of calcium ions using atomic absorption spectrophotometry with flame (AASF) after irrigation. He concluded that 15% EDTA, 0.2% chitosan and 10% citric acid effectively removed smear layer from the middle and apical thirds of the root canal. 15% EDTA and 0.2% chitosan were associated with the greatest effect on root dentin demineralization, followed by 10% citric acid and 1% acetic acid.

Kirchhoff et al (2014)[37] designed a study to evaluate the smear layer removal and quantify the calcium ion release resulting from final irrigation with different chelating solutions (apple *vinegar*, 5% malic acid, 5% acetic acid, 17% EDTA). The concentration of calcium ions released was analyzed by atomic absorption

spectrometry while smear layer removal was assessed in the cervical, middle, and apical thirds by SEM. It was concluded that 17% EDTA enabled greater smear layer removal and promoted release of the highest concentrations of calcium ions than the other solutions tested.

Darrag AM et al (2014)[38] compared smear layer removal efficacy after root canal final irrigation with 17% EDTA, 10% citric acid, Biopure MTAD, and 0.2% chitosan solutions. Samples were examined under scanning electron microscope for smear layer presence at coronal, middle and apical root canal levels. Results showed that smear layer removal at the middle and apical thirds was more effective when final irrigation was performed using 0.2% chitosan solution with significantly lowest mean ranks of scores compared to all tested groups except MTAD solution at the apical levels.

Chande KP et al (2014)[39] evaluated and compared the decalcifying effect of 17% ethylenediaminetetraacetic acid (EDTA), 15% citric acid, 37% phosphoric acid and 5.25% sodium hypochlorite on root canal dentin for three time periods (5, 10, and 15 minutes). The concentration of Ca2+ extracted from the dentin was measured by mass spectrophometry. It can be concluded that the use of solutions of 17% EDTA, 15% citric acid or 37% phosphoric acid produces root dentin decalcification, mainly during the first 5 min of action. The efficacy of 17% EDTA and 15% citric acid solutions was significantly higher than that of 5% phosphoric acid solution in all three immersion periods. Also, 5.25% sodium hypochlorite solution is capable of extracting small amounts of calcium from root dentin initially.

Taneja S et al (2014)[40] assessed the effect of irrigation with different chelating agents on the calcium loss and its subsequent effect on the microhardness of the root dentin. The chelating agents used were: 17% ethylenediaminetetraacetic acid (EDTA), 2.25% Peracetic acid (PAA) and QMix each with sodium hypochlorite. The calcium loss of the samples was evaluated using the Atomic Absorption Spectrophotometer followed by determination of their microhardness using Vickers Hardness Tester.

It was concluded that: Irrigation with 5% NaOCl + 2.25% PAA caused the maximum calcium loss from root dentin and minimum microhardness. Irrigation with 5% NaOCl + Distilled water caused minimum calcium loss from root dentin and maximum microhardness. A reduction in the microhardness of root dentin was observed with increase in calcium loss from root dentin.

Poggio et al (2015)[41] carried out a study to evaluate and compare the decalcifying capability of different irrigating solutions (Tubuliclean, Largal Ultra, ethylene diamine tetraacetic acid 17%, Tetraclean, Tetraclean NA), at three successive 5‐min interval immersion times, the concentration of calcium extracted from root canal dentin was assessed with an inductively coupled plasma‐atomic emission spectrometer.

Results showed that, the maximum amount of Ca2+ extracted from root canal dentin samples was reached after 10 min contact time except for citric acid‐based agents (Tetraclean and Tetraclean NA) which induced a higher and still increasing calcium release even after 10 min contact time. Also, it was suggested that in order to

obtain an efficient decalcifying action on dentin and to facilitate the biomechanical procedures, citric acid‐based irrigants can be applied.

Kolosowski KP et al (2015)[42] qualitatively evaluated the chemical characteristics of dentin surface and compared it with dentin exposed to NaOCl, EDTA, or CHX using the time-of-flight secondary ion mass spectrometry. Four blocks of dentin from a root of a human maxillary molar were embedded in resin and trimmed with a microtome to expose the dentin. Samples were randomly assigned to 4 treatment groups: (1) no irrigation treatment (sample A), (2) 2.5% NaOCl (sample B), (3) 17% EDTA (sample C), and (4) 2% CHX (sample D). Dentin surfaces were analyzed by time of- flight secondary ion mass spectrometry, which allowed characterization of dentin surface chemistry by both imaging and mass spectroscopic analysis obtained in high mass and spatial resolution modes.

Results showed that Sample A (NT) in positive ion analysis of dentin revealed intense peaks of Na+, K+ , Ca+ , and CH4N+ . And the negative ion analysis of NT dentin revealed intense peaks of CN, CNO, PO2 and PO3. In Sample B (NaOCl). The positive ion analysis of NaOCl-treated dentin showed an intense peak of Na+ with decreased detection of K+, Ca+, CaOH+ , and Mg+ and severely decreased CH4N+ compared with NT. Negative ion analysis of sample B dentin (NaOCl) revealed relatively unchanged peak distribution when compared with NT, except for the increased intensity, indicating the presence of Cl. In Sample C (EDTA). The positive ion analysis of EDTA-treated dentin showed an intense Na+ peak and less intense K+, CaOH+, and CH4N+ than NT. Ca+ and Mg+ were not detected in significant amounts. Additionally, in negative ion analysis of sample C (EDTA), PO2 and PO3

detection was decreased, with relatively unchanged intensity of CN, CNO, F and HCO2. In Sample D (CHX) compared with NT, CHX-treated dentin showed a slight decrease in K+, Ca+, and CaOH+ with unchanged intensity of Na+ , Mg+, and CH4N+ in positive ion analysis. There was also an appearance of peaks characteristic of CHX in this case. In the negative ion analysis, sample D(CHX) displayed an increased intensity of Cl and relatively unchanged intensities of peaks attributable to CN, CNO, PO2, PO3, F and HCO2.

Saha SG et al (2017)[43] evaluated the effect of various endodontic irrigants on the micro-hardness of the root canal dentin. Eighty freshly extracted mandibular premolars with single canal were selected and samples were divided into four groups based on the irrigants in which they were immersed i.e., 3% Sodium Hypochlorite (3% NaOCl), 17% Ethylene Dioxide Tetra Acetic Acid (17% EDTA), 0.2% Chitosan and 6% Morindacitrifolia Juice (MCJ) for 15 minutes each. All the specimens were then subjected to micro-hardness testing using a Vickers micro-hardness tester. Results indicated that in 17% EDTA and 0.2% Chitosan groups showed statistically significant reduction in the mean hardness of teeth after treatment, while no change was seen in the 6% Morinda citrifolia juice group and 3% NaOCl.

MATERIALS AND METHOD

MATERIALS AND METHOD

Sixty freshly extracted human mature mandibular premolars extracted for orthodontic or periodontal purpose were selected and stored in buffered saline solution as per the recommendations and guidelines by OSHA and CDC guidelines.

INSTITUTIONAL ETHICAL COMMITTEE

The study was approved by the Institutional Ethical Committee.

SELECTION CRITERIA

INCLUSION CRITERIA

1. Sound mandibular premolars

2. Fully formed apex (Closed apex).

3. Extraction for orthodontic or periodontal purpose purposes

EXCLUSION CRITERIA

1. Teeth with caries

2. Fracture

3. Teeth with defects

4. Developmental anomalies

5. Dehydration

ARMAMENTARIUM

Instruments and Equipment:

- Hand instrument (GDC, India) (PLATE I)
- Cotton holder and waste receiver (PLATE I)
- Hand Scaler (Satelec P5 Newtron Worktop Scaler, Satelec Acteon)
- Digital Vernier calliper (WorkZone Hand Tools, Germany) (PLATE I)
- Digital Radiovisiography System (Kodak 5100 RVG, France)
- Diamond disc (PLATE I)
- Tooth brush
- Straight hand-piece (Marathon, Japan) (PLATE I)
- Magnetic stirrer (PLATE I)
- Precision Weighing Balances (Sartorius) (PLATE I)
- Borosil beakers
- Test tubes
- Thermocycling unit. (LG, India) (PLATE VI)

- Microhardness Testing Machine (Reichert, Austria) (PLATE VII)
- Inductively coupled plasma -Atomic absorption spectrometer (Spectro Analytical instrument, Germany) (PLATE VII)

Materials:

- 5ml syringe with 24 gauge needle (Dispovan HMD, India) (PLATE III)
- Irrigating solution – Normal saline (0.9 % w/v, Nirlife, India) (PLATE III)
- Auto polymerized self-cure clear acrylic (DPI, India) (PLATE III)
- Chelating agents
    - EDTA salts (Kims chemicals, India) (PLATE II)
    - Citric acid salts (Kims chemicals, India) (PLATE II)
    - Chitosan powder (Research-Lab fine chem industry, India) (PLATE II)
- 1% glacial acetic acid (Thermo electron LLS, India) (PLATE II)
- Distilled water (Alfa chem laboratory, India) (PLATE II)
- Varnish

PREPARATION OF SAMPLES

The soft tissue attached to the root surfaces was removed using gauze piece and a fine brush. All the samples were radiographed with RVG system (Kodak 5100 RVG, France) to confirm the presence of single canal. To standardize the size of the samples, the teeth were decoronated at the cemento-enamel junction and at the apical

end of the root using a diamond disc under copious water irrigation to obtain 10 ± 1 mm length which was confirmed using a digital vernier caliper (WorkZone Hand Tools, Germany). This length was standardized for all the specimen in different groups.

DISTRIBUTION OF STUDY GROUPS:

The teeth were then randomly divided into four groups, each group containing 15 specimens (n=15).

GROUPS	**SOLUTION**
Group 1	Distilled water (Control group)
Group 2	17% EDTA
Group 3	15% Citric acid
Group 4	0.2% Chitosan

Teeth were bisected longitudinally and one half of the specimen was used for calcium analysis and other half for microhardness testing. Pulp tissue was removed and two consecutive layers of nail varnish was applied on cementum and dentin surface leaving only a 2 mm window exposed.

PREPARATION OF TEST SOLUTION

GROUP 2 (17% EDTA SOLUTION)

17 grams of EDTA salt was added in 100ml of distilled water in a magnetic stirrer bath for 1 hour.

GROUP 3 (15% CITRIC ACID SOLUTION)

15 grams of citric acid salt was added in 100ml of distilled water in a magnetic stirrer bath for 1 hour.

GROUP 4 (0.2% CHITOSAN SOLUTION)

0.2 grams of chitosan was added in 100ml of distilled water with 1% of acetic acid in a magnetic stirrer bath for 1 hour.

The samples were immersed in 10 ml of the test solution for a duration of 2 minutes as per the groups. The time was carefully monitored using stopwatch and samples were removed from the solution after 2 minutes.

The samples were then subjected to a thermocycling regimen of 500 cycles at 5°C and 55°C with a dwell time of 30 seconds and a transfer time of 10 seconds. These temperature parameters were chosen as the teeth are usually subjected to sudden temperature variations in this temperature range.

One half of the split section was used to determine calcium loss using Inductively Coupled Plasma -Atomic Absorption Spectrometer (Spectro Analytical instrument, Germany), while the other half was mounted on the acrylic block for evaluating the micro-hardness using Vickers Microhardness Testing Machine (Reichert, Austria) after subjecting the samples to test solutions for 2 minutes.

DETERMINATION OF CALCIUM LOSS

The initial or a baseline calcium concentration of each solution was analyzed using ICP‑AES (Spectro Analytical instrument, Germany). In each group, the specimen was individually immersed in 10ml of test solution for 2 minutes as per the

groups. Then the samples were taken out of the test tube after 2 minutes. Accordingly, 2ml of the solution was aspirated from the bath after 2 minutes for Calcium Analysis. The Calcium level of each group was determined by Inductively Coupled Plasma-Atomic Emission Spectroscopy (Spectro Analytical instrument, Germany)**.**

A test method similar to that previously reported by Machado-Silveiro et al[25] was used to evaluate the mineral content of root canal dentin with the use of ICP-AES. In this study, 3 measurements were performed for each solution to increase measurement sensitivity. The means of the measurements were calculated as ppm (parts per million) by a software.

All decalcification procedures were carried out on the same day at the same room temperature because an increase or decrease in temperature can alter the demineralization process. The differences in mineral content between the groups were analyzed and the results were statistically evaluated.

DETERMINATION OF MICROHARDNESS ROOT DENTIN

The other half of each sectioned specimen was immersed in 10 ml of the test solution for a duration of 2 minutes as per the groups and were mounted on acrylic block. The microhardness of the each specimen was evaluated at different dentin surface using a microhardness testing machine (Reichert, Austria). All experiments were completed under the same conditions: 50 g load and 15s dwell time, following the guidelines given by Cruzfilho et al.[15] In each sample, three indentations were made in the middle third of the root canal in the window created with varnish.

The diamond shaped indentations were carefully observed in an optical microscope with a digital camera and image analysis software, allowing the accurate digital measurements of their diagonals. The average length of the two diagonals was used to calculate the microhardness value (MHV). The representative hardness value for each sample was obtained as the mean of the three indentations values and the results were statistically evaluated.

The data was collected and tabulated using an excel sheet (Microsoft Office 2013).The data on calcium loss and hardness was obtained on sample treated with EDTA, citric acid and chitosan and statistically evaluated using SPSS ver 20.0 (IBM Corp.) software and statistical significance was tested at 5% level.

ALGORITHM FOR METHODOLOGY

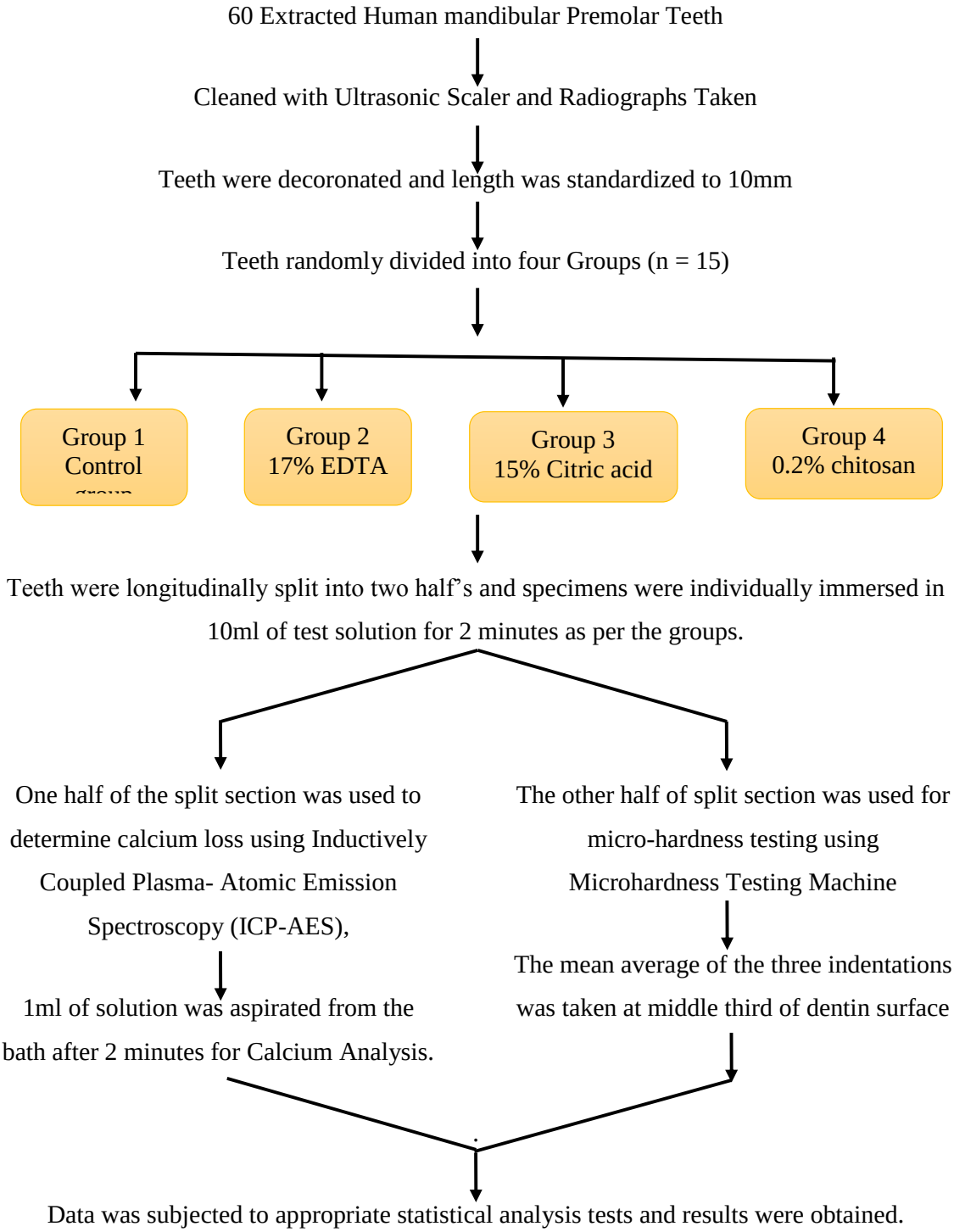
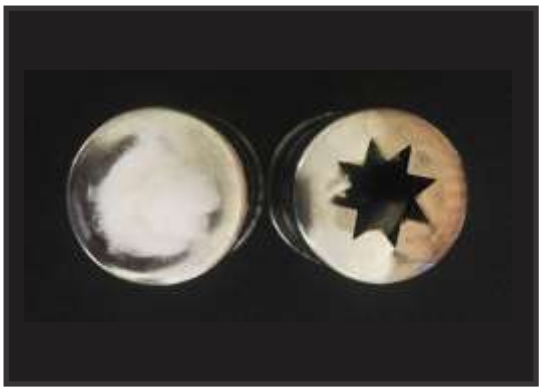


PLATE - I

ARMAMENTARIUM

Hand instruments
(GDC, India)



Cotton Holder & Waste receiver

Digital Vernier Caliper
(Workzone tools, Germany)

Straight hand (Marathon, Japan piece & Diamond disk

Precision weight balance

Magnetic stirer

PLATE - II

MATERIALS

Distilled water

EDTA salt (Kims Chemicals)

Citric acid salt (Kims Chemicals)

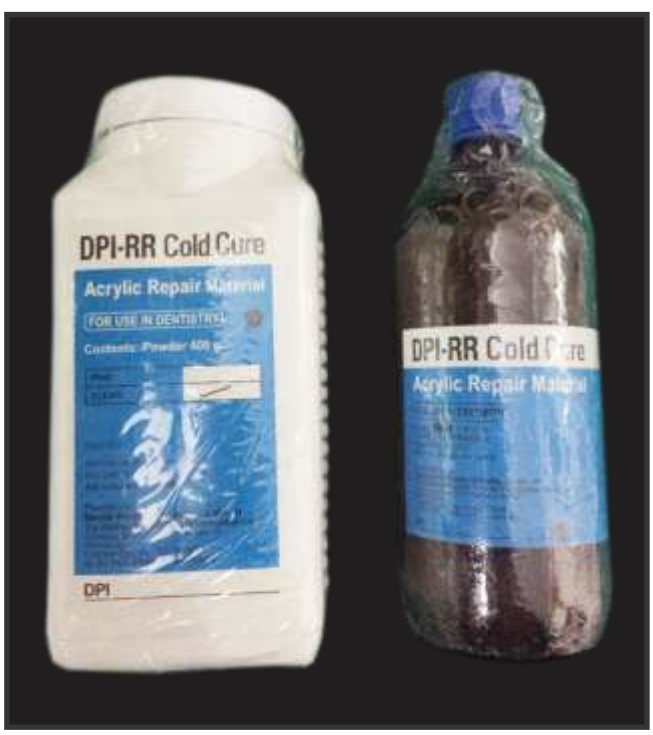
1% Acetic Acid (Fishers Scientific)

Chitosan Powder (Research Lab)

PLATE - III

MATERIALS

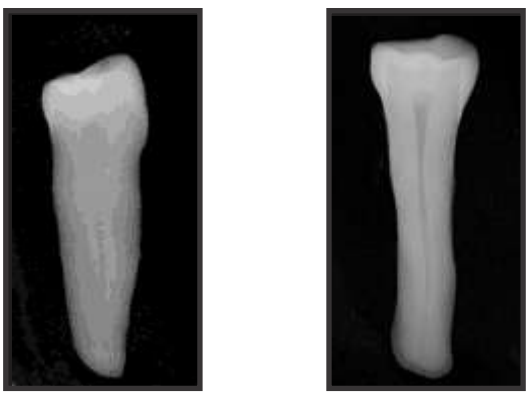
0.9% w/v Normal Saline (Nirlife, India)
Irrigating Syringe (DispoVan HMD, India)



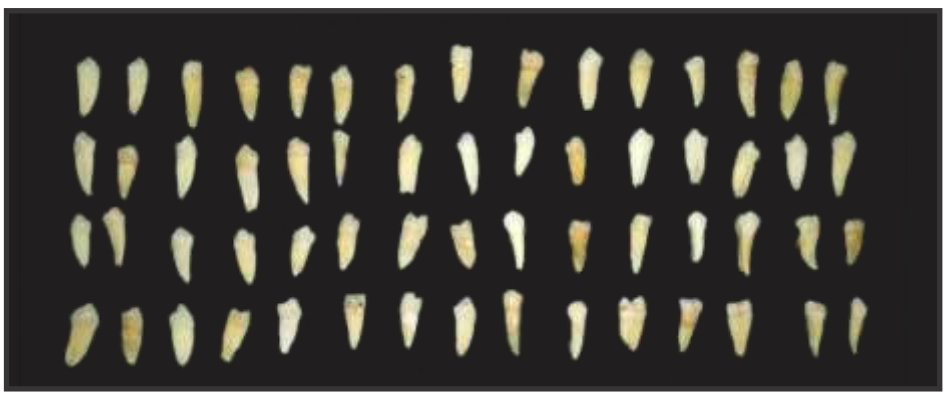
Autopolymerized Acrylic Resin (DPI-RR Cold Cure)

PLATE - IV

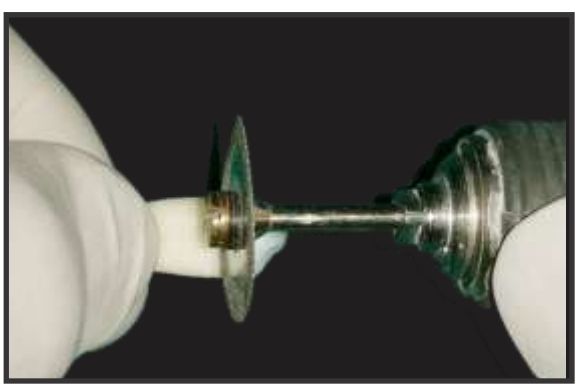
METHOD



Pre Operative Radiographs



Total sample size (60 samples)

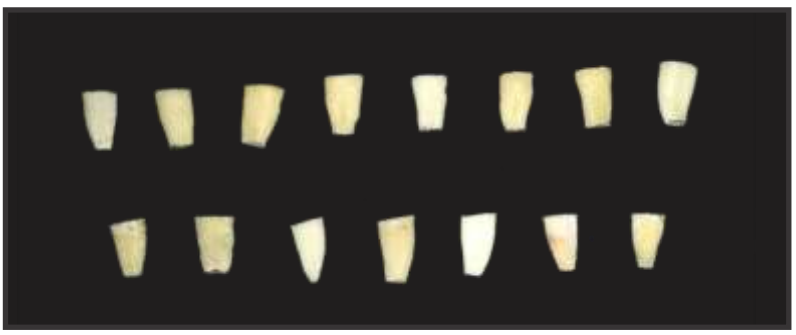


Decoronation of samples

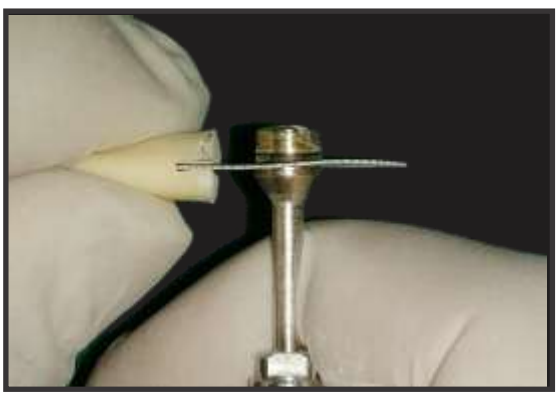
Sample size standardization

PLATE - V

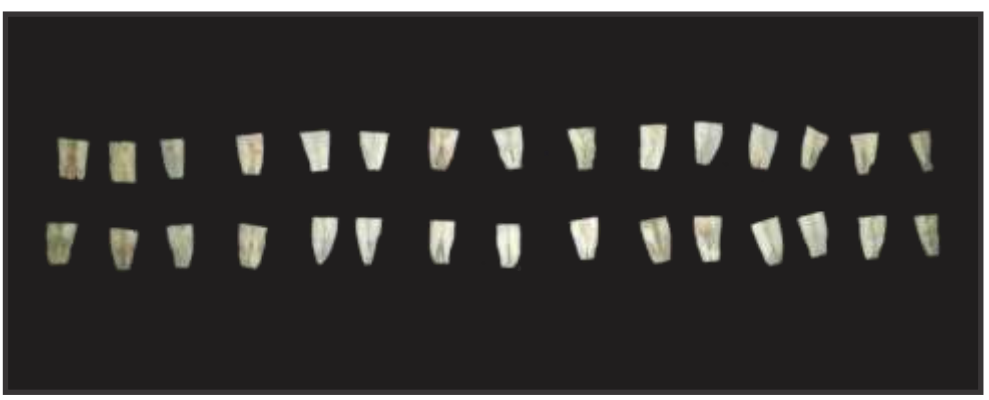
METHOD



Decoronated samples per group

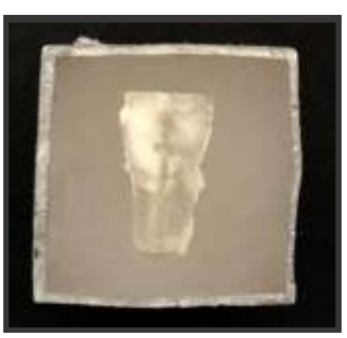


Longitudinal sectioning of samples

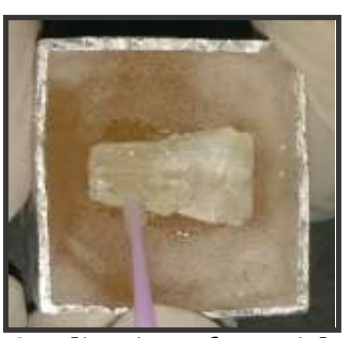


Longitudinal sections of samples per group

Mould 15x15mm



Acrylic mould



Application of varnish

PLATE - VI

METHOD

THERMOCYCLING OF SAMPLES

Thermocycling Unit, (LG, India)

PREPARATION OF CHELATING SOLUTIONS

Weighing of salts in precision balance

Salt was added in 100ml of distilled water in a magnetic stirrer bath for 1 hour

PLATE - VII

METHOD

Sample immersion in 10ml of chelating agents for 2 minutes

TESTING OF SAMPLES

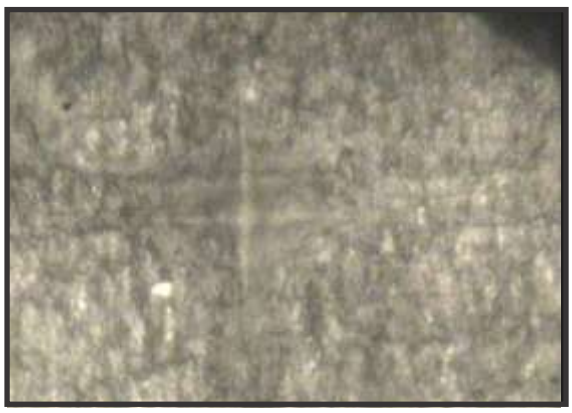
Inductively Coupled Plasma Atomic Absorption Spectrometer (ICP-AES, Spectro Analytical Instrument, Germany)

Aspiration of solution

Vickers microhardness testing (Reichert, Austria)

PLATE - VIII

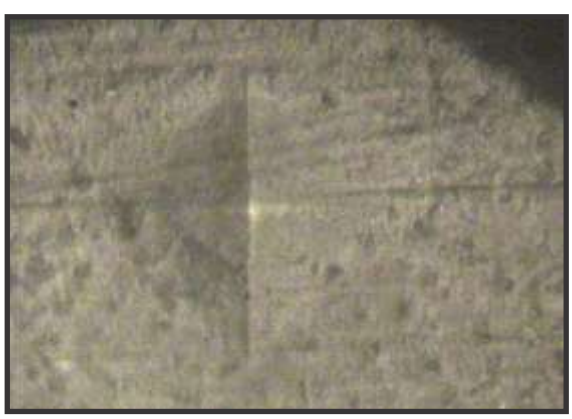
RESULT



GROUP 1

GROUP 2

GROUP 3



GROUP 4

RESULTS

RESULTS

This study was performed to determine the effect various chelating agents i.e. 17% EDTA, 15% Citric acid and 0.2% Chitosan on amount of calcium loss and reduction in microhardness of root dentin when exposed for 2 minutes and the results were then compared among themselves. The four group in this study were as follows:

Group 1 – Distilled water (control group)

Group 2 – 17 % EDTA

Group 3 – 15% Citric acid

Group 4 – 0.2% Chitosan

All samples were immersed in chelating agents in their respective groups for 2 minutes followed by mechanical testing for calcium loss and microhardness using Inductively Coupled Plasma- Atomic Absorption spectrometer (ICP-AES) and Vickers microhardness tester respectively. The amount of calcium loss was measured in ppm and microhardness was measured in Vickers hardness number (VHN).

Statistical methods

The data on calcium loss and hardness was obtained on sample treated with EDTA, citric acid and chitosan. Descriptive statistics of parameters like mean, median, standard deviation and 95% CI were obtained for each group. One-way analysis of variance was performed to determine the statistical significance of difference in the means of parameters. Further, Tukey's post-hoc test was performed to determine the pair wise significance of difference between the means. The analysis was performed independently for ca loss and hardness. The correlation between calcium loss and hardness was obtained using Pearson's correlation coefficient. All the analyses were performed using SPSS ver 20.0 (IBM Corp.) software and statistical significance was tested at 5% level.

The description of methods used in the study are as below:

One-way Analysis of variance

Analysis of variance (ANOVA) is used to test the significance of difference in the mean of three or more groups. The basic assumption is that the variable of interest is normally distributed in the population under study.

Method

Here the interest is to test the null hypothesis that the population means are same, i.e.

$H_0 : \mu_1 = \mu_2 = ...\mu_m$ against the alternative H_1 that they are not same.

Some of the statistics computed to test the hypothesis are as below:

i) **Grand mean**: It is the mean of set of all observations in the studied groups and is given by:

$$\bar{x}_{GM} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

ii) **Total sum of squares:** It is the sum of squares of each observation from the grand mean and is given by:

$$TSS = \sum_{i=1}^{N} (x_i - \bar{x}_{GM})^2$$

Total sums of squares is the sum of two components i.e., variation between groups and within groups.

iii) **Between group sum of squares**

$$SSB = \sum_{j=1}^{m} n_j (\bar{x}_j - \bar{x}_{GM})^2$$

iv) **Within group sum of squares**

$$SSW = \sum_{j=1}^{m}\sum_{i=1}^{n} (x_{ij} - \bar{x}_j)^2$$

The mean sum of squares is obtained by dividing the above sum of squares with the respective degrees of freedom, i.e. N-1, p-1 and $p(n$-1).

v) F-statistic: It is the ratio of between and within mean sum of squares

$$F = \frac{MS_{Between}}{MS_{Within}}$$

If the *p*-value based on F-statistic is greater than 0.05, H_0 is accepted, otherwise H_1 is accepted.

vi) Tukey's post-hoc test

After performing ANOVA, if alternative hypothesis H_1 is accepted, then the subsequent interest is to determine the pair wise significance of difference in the means of study groups. This could be carried using Tukey's post-hoc test. The difference between the means of all groups are determined and compared with this critical difference called the honest significant difference (HSD). It is given by: $HSD = q\sqrt{\frac{MS_{within}}{n}}$

where, *q* is the studentized range statistic derived from the tables, *n* is the sample size and the mean square value is from the ANOVA analysis. If the critical difference exceeds the absolute difference between any two sample means, then the corresponding means differ significantly.

Pearson's correlation

Pearson's correlation coefficient quantifies the relationship between two measurable variables. It measures the linear relationship between two variables. Thus, if *X* and *Y* are two variables taking values x_1, x_2,…x_n and y_1, y_2,…y_n, then the correlation coefficient (*r*)between the two variables is given by:

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[\,n\Sigma x^2 - (\Sigma x)^2\,]\,[\,n\Sigma y^2 - (\Sigma y)^2\,]}}$$

The value of *r* lies between -1 to +1, with -1 indicating perfect negative correlation and +1 indicating perfect positive correlation.

The mean and median value of calcium loss and standard deviation of different groups with 95% Confidence interval are presented in Table no. 1.

In Group 1 (Control group), i.e, where the teeth were immersed in distilled water for 2 minutes, mean and median value of calcium loss observed were 0.29 and 0.26 respectively and standard deviation was 0.12 and 95% Confidence interval was obtained at 0.22 – 0.35.

In Group 2, i.e, where the teeth were immersed in 17% EDTA for 2 minutes, mean and median value of calcium loss observed were 35.08 and 26.30 respectively and standard deviation was 22.22 and 95% Confidence interval was obtained at 24.08 – 46.08.

In Group 3, i.e, where the teeth were immersed in 15% Citric acid for 2 minutes, mean and median value of calcium loss observed were 50.95 and 46.26 respectively and standard deviation was 18.87 and 95% Confidence interval was obtained at 41.45 – 60.45.

In Group 4, i.e, where the teeth were immersed in 0.2% Chitosan for 2 minutes, mean and median value of calcium loss observed were 16.20 and 16.59 respectively and standard deviation was 2.15 and 95% Confidence interval was obtained at 15.10 – 17.30.

One-way analysis of variance was applied to compare the mean for Calcium loss across four groups. The resulting F-value was 34.20, with a corresponding *P*-

value < 0.0001, indicating statistically significant difference in the mean *Ca* loss across four groups. (Table 2A)

Since there exists significant difference across groups, pair wise comparison of mean values between the four groups was carried out using TukeyHSD as shown in Table 2B.

It suggests that highly significant difference exists between Control and all other groups by the p-value <0.0001. This indicates that all chelating agents were potent in extracting calcium from root dentin. Further, the difference between Group 2 and Group 3 or Group 4 were also significant as indicated by the p-values 0.0218 and 0.0044 respectively. This indicates that though all chelating agents were potent in removing calcium from root dentin there was statistically significant difference between them. The difference between Group 3 acid and Group 4 was also statistically highly significant with p < 0.0001. This indicates that, though 0.2% Chitosan was able to extract calcium from root dentin, it was not as potent as 15% Citric acid.

This results suggest that 15% Citric Acid is most potent in extracting calcium from root dentin followed by 17% EDTA group and 0.2% Chitosan group and distilled water showed minimum calcium loss when compared with other group.

Figure 1 show the graphical visualization of mean values along with pair wise comparison between the groups for Calcium loss.

The mean and median value of microhardness test and standard deviation of different groups with 95% Confidence interval are presented in Table no. 3.

In Group 1 (Distilled water), mean and median value of microhardness observed were 71.51 and 72.42 respectively and standard deviation was 5.70 and 95% Confidence interval was obtained at 68.61 – 74.41.

In Group 2, (17% EDTA) mean and median value of microhardness observed were 51.39 and 51.39 respectively and standard deviation was 1.96 and 95% Confidence interval was obtained at 50.40 – 52.38.

In Group 3 (15% Citric acid), mean and median value of microhardness observed were 49.64 and 49.11 respectively and standard deviation was 1.47 and 95% Confidence interval was obtained at 48.9 – 50.38.

In Group 4 (0.2% Chitosan), mean and median value of microhardness observed were 57.06 and 55.50 respectively and standard deviation was 3.98 and 95% Confidence interval was obtained at 55.06 – 59.06.

One-way analysis of variance was applied for Micro hardness across four groups. The resulting F-value was 108.9, with corresponding P-value < 0.0001. Statistically significant difference of mean micro hardness was observed across groups.

Since there exists significant difference between the groups, pair wise comparison of mean values between the four groups was carried out using TukeyHSD as seen in Table 4B.

It suggests that highly significant difference of mean micro hardness exists between Control and all other groups by the p-value <0.0001. This indicates that all chelating agents were potent in reducing microhardness of root dentin when compared with control group. Further, there was insignificant difference of mean micro hardness

exists between Group 2 and Group 3 by the p-value = 0.5647 whereas there was significant difference exists between Group 2 and Group 4 (p-value = 0.0005). This indicates that EDTA and Citric acid reduce microhardness with no significant difference between them while EDTA caused more reduction in microhardness when compared with 0.2% Chitosan. The difference between Group 3 and Group 4 were highly significant as indicated by the P-value < 0.0001. This suggest that 15% Citric acid more caused reduction in microhardness than 0.2 Chitosan.

This results suggest that 15% Citric acid caused maximum reduction in microhardness followed 0.2% Chitosan and 17% EDTA and distilled water group showed maximum microhardness when compared with other groups.

Figure 2 show the graphical visualization of mean values along with pair wise comparison between the groups for Micro hardness.

The correlation between calcium analysis and microhardness test was obtained using the Pearson's correlation test. Pearson's correlation coefficient obtained was -0.6904, which was highly significant with P-value < 0.0001. In other words, there is a strong negative correlation between calcium loss and hardness i.e. as the percent calcium loss increases, the hardness decreases.

Figure 3 shows the scatter plot showing the relationship between calcium loss and hardness.

DISCUSSION

DISCUSSION

Long term prognosis of the root canal treatment is entirely dependent on the quality of instrumentation, irrigation, disinfection and finally the obturation of the root canal system.[1] The probability of success in endodontic therapy increases as the debris and smear layer is removed from the root canal. The "smear layer" is formed on the root canal walls during biomechanical preparation. It contains inorganic and organic substances that include fragments of odontoblastic process, microorganisms and necrotic debris. Its presence increases microflora and the inorganic toxins, decreases the sealing ability and increases the potential for bacterial survival and reproduction.[44]

Various irrigants and chelating agents have been used to remove or modify the smear layer before obturating the root canal system. In endodontics, sodium

hypochlorite is the most regularly used irrigant for cleaning & disinfection of root canal system. It has many suitable qualities and properties as it performs bactericidal cytotoxicity, dissolution of organic material and lubrication. But sodium hypochlorite by itself is not sufficient for total cleaning of the endodontic system as it acts only on organic tissue and has minimal to no effect on smear layer.[44,45]

Thus, chelating agents are required for complete removal of smear layer. Chelators were introduced into endodontics by Nygard-Ostby in 1957 to chemically soften the root canal dentin and dissolve the smear layer as well as increase dentin permeability. Various chelating agents used in endodontics are EDTA, Citric acid, Phytic acid, Apple vinegar, Etidronate, etc.

Currently, all the products in the dental market sold to dissolve smear layer are based on ethylenediaminetetraacetic acid (EDTA) or citric acid. Von der Fehr and Nygaard-Ostby (1963) introduced a combination of Cetavlon and EDTA, known as EDTAC. Stewart et al (1969) added urea peroxide to EDTA, which helps in lifting debris out of the root canal. Ruddle et al (2002) incorporated hypaque, a high contrast injectable dye in 17% EDTA, visualise the complexity of the root canal system. Torabinejad presented MTAD, a mixture of citric acid, doxycycline and Tween 80, to disinfect root canals and remove smear layer. And Tetraclean which is a Citric acid based irrigants and contains Doxycycline (50 mg/5 ml) and Polypropylene glycol.[44]

Recently, Chitosan, a natural derivative and Morinda Citrifolia, Juice, a herbal derivative are gaining acceptance in various endodontic research as a chelating agents. Saha SG in their study found that the use of 6% Morinda Citrifolia Juice and 3%

NaOCl does not significantly affect the dentin micro-hardness in contrast to 17 % EDTA and 0.2% Chitosan.[43]

Most researchers have highlighted only the ability of chelating solutions to remove the smear layer but their potential deleterious effect on the mechanical properties of dentin is also important. It has already been established that these chemicals used for endodontic irrigation are capable of causing alterations in the chemical composition of dentin. In particular, chelating solutions might play a part in influencing the physical and mechanical properties of dentin.

Dentin consists of both organic and inorganic components. The calcium ions (Ca^{2+}) present in hydroxyapatite ($Ca_3(PO_4)_5OH$) crystals is one of the main inorganic elements of dentin. Any change in the Ca/P ratio may in turn change the microhardness, permeability and solubility of dentin and may also adversely affect the sealing ability and adhesion of dental materials such as resin-based cements and root canal sealers to dentin.[21] Indeed, dentin adhesion depends on the presence of residual Calcium on the bonding area, and there is evidence that partial depletion of surface Calcium may significantly reduce the bond strength of some adhesive materials.[47] It has been indicated that microhardness determination can provide indirect evidence of mineral loss or gain in dental hard tissues. The significant alteration in dentin hardness after irrigation with different chemicals indicates the direct effect of these chemical solutions on the components of dentin structure.

Studies on the softening effect of EDTA have given controversial results. Von der Fehr and Nygaard-Ostby[48] described a depth of demineralisation of 20 to 30 μm

after 5 min working time of EDTAC. A measurable softening effect of EDTA on dentin was confirmed by the studies of Fromme et al[49] and Pawlicka et al[50].

Controversially, Fraser et al came to the conclusion that there was a softening effect in three different chelators, among which RC-Prep could be found only in the coronal and middle third of the root canal but not in the apical part. These findings were confirmed by a further study using Largal Ultra (Septodont, France), a liquid chelating agent containing EDTA and cetrimide.[49]

Scientific literature lacks evidence on comparison of the effect of EDTA, Citric acid and Chitosan on calcium loss and their effect on microhardness of root dentin. Therefore, the present study has been undertaken to evaluate the effect of different irrigation regimens on calcium loss and its effect on the micro-hardness of the root dentin.

In the present study, single rooted premolars were selected as it is easily available after orthodontic extraction. Thus, evaluation of effect of chelating agents on calcium loss and reduction in microhardness of root dentin can give idea about the strength and durability of tooth after endodontic treatment.

Sample size was studied referring to the article by Mishra L. et al. (2012), the data on mean Calcium loss and reduction in microhardness was referred for different treatment categories. The mean and SD values for different groups resulted into an effect size of 0.949 with one-way analysis of variance. However, considering this effect size to be larger, a more stringent effect size of 0.5 was considered for the proposed study, which resulted into a sample of 12 per group that can provide the

desired effect with 80% power and 95% confidence. Finally, a sample of 15 per group (Total: 60) was considered for the study.

60 mandibular premolars with single root were checked for extra canals, open apex or any surface defects using radiographic and microscopic examination and were collected, stored in buffered saline solution, disinfected, and handled as per the recommendations and guidelines laid down by Centers for Disease Control and Prevention (CDC). And then were divided into 4 similar groups under stratified randomization.

To minimize the error and standardized the size of the samples, the teeth were decoronated at the cemento-enamel junction and at apical end of the root using to obtain 10 ± 1 mm length. This is the same protocol that has been used by Sayin TC (2007).[31] Masiero AV and Barletta FB (2005) who used decoronation to ensure standardization of specimen length allowing a more reliable comparison.[51]

In the present study, the action of chelating agents on calcium loss and microhardness was studied on the roots were split longitudinally instead of cut transversally into discs. under clinical conditions, it is evident that during canal irrigation the solution initially enters in direct contact with the most superficial dentin layer of the root canal lumen and then diffuses through the dentinal tubule. Berutti E et al reported that the irrigants diffuses through the dentin tubules from the main root canal to a distance of up to 130 µm.[52] Therefore, it seems more accurate to evaluate the action of chelating agents on the longitudinal sections.

In the present study, the root canals were not prepared prior to analysis; thus, no smear was present on the dentin surface. The rationale behind omitting this step was to enable measurement of Calcium loss that occurred solely on intact root dentin, whereas avoiding any possible "contamination" of readings that could have been caused by the calcium incorporated into the loosely bound smear.

Also, two consecutive layer of varnish was applied on the specimen on cementum and dentin, leaving only 2 mm of dentin surface exposed. This was done to attain more clinical relevance as only root canal surface is exposed to chelating agent. This could give more accurate results on amount of calcium extracted from root dentin.

Ethylenediaminetetraacetic acid (EDTA) is available in variety of formulation. Its reaction with calcium ions in dentin results in calcium chelation, promoting decalcification of dentin at approximate depths of 20–30 μm within 5 min.[48] It has been reported to as an excellent agent to remove smear layer from the root canal. Therefore, EDTA has been used as a gold standard against which other chelating agents can be compared.

However, the demineralizing effect of EDTA acts concurrently and indistinguishably on the smear layer and root dentin, with consequent exposure of collagen and reduction of dentin microhardness. Sayin et al verified that the use of EDTA, either alone or combined with sodium hypochlorite, reduces the microhardness of root dentin significantly. The reducing effect of EDTA can be felt in the first minute of use, and is directly proportional to the time of application.[31]

Another chelating agent used for removing the smear layer from root canals is citric acid. This agent is a weak organic acid with a less cytotoxic effect than EDTA. Prior studies have shown that citric acid removes calcium ions efficiently from the dentin. However, in relation to the effect on dentinal microhardness, it has been reported that 10% citric acid is less efficient than 17% EDTA in 1-, 3- and 5-min periods of action.[18]

Chitosan, a natural polysaccharide is biocompatible, biodegradable, and bioadhesive. It has chelating ability and lacks toxicity. In dentistry, its addition to calcium hydroxide paste as an intracanal medication has been shown to promote prolonged calcium ion release. 0.2% chitosan in combination with ultrasonics performed better than 17% EDTA in the removal of oil-based calcium hydroxide.[14] Silva PV *et al.* found 0.2% chitosan to be equally effective to 15% EDTA and 10% citric acid in smear layer removal and microhardness reduction.

It has been important to test the effect of irrigation solutions on all dentin tissues, because contact might occur during irrigation procedures. There is no consensus on the time a decalcifying agent must be in contact with the root canal wall to adequately remove smear layer; reports vary from 1 to 15 min. Patterson (1963) stated that the main effect of the chelator substances occurred after 5 min of application. Hulsmann et al extended the experimental time to 10 min.[5] EDTA has been reported to remove smear layer in 1 min if the fluid is able to reach the root canal wall surface.[46] However, it has also been suggested that the fluid should be kept in the root canal for at least 15 min to obtain optimal results.[5] Haapasalo et al (2010) suggested that aqueous chelators are advised to be used for 2-3 minutes for the final

irrigation.[1] In the present study, chelating agents was used for 2 min, a more realistic time in terms of clinical practice.

To simulate the oral conditions more closely, the teeth were subjected to a thermocycling regimen of 500 cycles between the temperature range of 5°C and 55°C with a dwell time of 30 s at each temperature setting and a 10 s transfer time. This is the same protocol that has been used by Kalburge et al (2013).[53] This is because the restorations are subjected to extreme temperature changes within the oral cavity that usually range between these values. Since the teeth are in contact with this hot/ cold foodstuff for only a very short period of time, a short dwell time was selected. This regimen duplicates the aging process for the tooth as well as the restoration and hence, was included in the methodology. This is in accordance with a study by Gale and Darvell in 1999 who recommended these parameters for thermocycling. These parameters have also been widely accepted by various authors for simulating the aging process.[54]

For standardization, all samples were immersed in the selected irrigating solutions for 2 minutes. To precisely assess the amount of released Calcium, accuracy of the applied technique is of great importance. In the present study, ICP- AES was used to determine calcium loss from the root dentin. As with ICP-AES, the mineral content can be detected at concentrations of parts per billion (_g/L). In addition, multiple elements can be measured at the same time with this technique. However, other techniques like scanning electron microscopy and energy dispersive spectrometry can also measure the mineral content with amounts detectable at concentrations of parts per million (mg/L).

In present study, sample were immersed in 10ml of chelating agents for 2 minutes after which 2ml of solution was aspirated by ICP-AES machine as test method similar to that previously reported by Machado-Silveiro et al.[25]

Micro-hardness testing, is widely used to study fine scale changes in the hardness, either intentional or accidental and is one of the most uncomplicated and non-destructive methods. It is the gold standard parameter to observe alterations of mechanical properties in mineralized tissues and a good predictor of the other important mechanical properties such as Young's modulus and yield stress.[55]

Nanoindentation technique, which is the latest technology in hardness measurement, has been developed to investigate surfaces at smaller scales, especially for thin surface coatings and films and only the thin superficial layer of tissues can be examined. The conventional microhardness assessment is done by either Vickers or Knoop tests. While Knoop indenter is able to reveal deformations 20 μm in length, as small as 5 μm area in diameter can be measured by Vickers indenter; in other words, Vickers indenter is more sensitive to plastic deformations.[55] Hence, in the present study, Vickers microhardness test was used to determine the loss of microhardness of root dentin after its immersion into root dentin.

In the present study, at 0.5mm level from the root canal space and the mid root dentin region was selected in order to minimize structural variations and also to obtain uniform baseline results for evaluation. Ground polishing of the samples were done to eliminate any surface irregularities and to obtain a mirror like finish.[55] The glossy surface ensures the reflection of light so that indentations can be clearly visualized when testing with the VHN testing machine.

The reduction in mineral content and microhardness between the groups were analyzed and the results were statistically evaluated using One-way ANOVA and Tukey's post-hoc test. And the correlation between calcium loss and hardness was obtained using Pearson's correlation coefficient.

For calcium loss analysis, in the Group 1 (distilled water) which served as a control group, showed the minimum mean value of calcium loss of 0.29 ± 0.12 ppm which was negligible.

In Group 2, in which all samples were immersed in 17% EDTA solution, showed the mean value of calcium loss of 35.08 ± 22.22. There is highly significant difference between the Group 1 and Group 2 ($P<0.0001$). This demonstrates that 17% EDTA has high decalcifying ability when compared with distilled water. This results are in accordance with previous study. Ari H & Erdemir A concluded that there was a significant decrease in the Calcium level after treatment with 17% EDTA. [62]

Nygaard- Ostby explained the demineralisation of the dental hard tissue by the EDTA solution. The mineral component of dentin is mainly of calcium and phosphate, which are soluble in water. Chelators such as EDTA form a stable complex with calcium. When the disodium salts of EDTA is added to the equilibrium, calcium ions are removed from the dentin into the solution. This leads to the dissolution of further ions from dentin.[3]

Perez et al[56] explained that, at neutral pH (7.4), EDTA showed chemically two co-existing reactions. They are complex formation and protonation. EDTA-HNa_3 is normal available form of EDTA and the reaction is as follows:

1) $EDTA\ H_{3-} + Ca_{2+} = EDTACa_{2} + H$

2) $EDTA\ H_{3-} + H = EDTAH$

As this reaction proceeds, the exchange of calcium from the dentin by hydrogen results in subsequent decrease in pH. Beacause of the release of acid, the efficiency of EDTA decreases with time and the reaction of the acid with the hydroxyapatite effects the solubility of dentin.

In Group 3, in which all samples were immersed in 15% Citric acid for 2 minutes, showed the mean value of calcium loss of 50.95 ± 18.87 ppm, which was highest from all the other groups. Statistical analysis showed highly significant difference in calcium removal when compared with the Group 1(Distilled water) ($P<0.0001$) and there was significant difference when compared with Group 3 ($P<0.0218$). This implies that 15% Citric acid was more potent in removing calcium from root dentin than distilled water and 17% EDTA.

This results are in accordance with Zehnder et al. who found that a 10% citric acid solution significantly removes more calcium than a 15.5% EDTA solution and 18% etidronate solution.[29] Machado-Silveiro et al reported that 1% and 10% citric acid promoted greater decalcification of dentin than 17% EDTA and 10% sodium citrate.[25] Whereas in contradiction, Spano et al[33] evaluated the concentration of calcium ions by using root canal chelators according to flame atomic absorption spectrometry and suggested that a solution of 15% EDTA is better at removing calcium ions than a solution of 10% citric acid. These could be due to difference in study design and concentration and pH of solution used to evaluate the calcium chelation property.

Under normal reactive conditions, the salt resulting from the reaction of citrate with calcium is formed at a 1:1.5 ratio, whereas the chelate formed by the union of the EDTA ion with a bivalent metallic cation (eg, Ca2+, Mg2+, and Zn2+) occurs at a 1:1 ratio (i.e., 1 mol of EDTA chelates 1 mol of metallic ions).[57] If both solutions were used at the same concentration, citric acid would remove more calcium ions, thus contributing to a greater reduction in dentin microhardness. In the present study, EDTA was used at a 17% concentration, whereas citric acid was used at a 15% concentration. Because the concentration of the solutions is almost the same, the citric acid was expected to remove more calcium ions from dentin than EDTA.[58]

In Group 4, in which all samples were immersed in 0.2% Chitosan for 2 minutes, showed the mean value of calcium loss of 16.20 ± 2.15 ppm. Statistical analysis showed significant difference in calcium removal when compared with the Group 1(Distilled water) ($P< 0.0214$) and Group 2 (EDTA) ($P<0.0044$) and there was highly significant difference when compared with Group 3(Citric Acid) ($P<0.0001$). No previous studies has evaluated the calcium chelation property of 0.2% chitosan.

The mechanism of action of 0.2% Chitosan is not yet clear, but it is thought that adsorption, ionic exchange and chelation property may be responsible for formation of the complex between substance and metallic ions. There are two theories of chitosan which explains its chelating process. They are:

1. Bridge model, which claims that two or more amino group of one Chitosan chain will bind to the same metallic ion.[59]

2. Only one amino group of the structure is involved in binding that is the metallic ion which is "anchored" to the amino group.[60]

On comparision to EDTA, the Chitosan polymer consists of a chain of many dimers of chitin. The dimers of chitin have two nitrogen atoms and two free electrons that are liable for the interaction of ions between the chelating agent and the metal.[61]

For microhardness analysis, in the Group 1 (Distilled water), showed the maximum hardness with mean value of 71.51 ± 5.70 VHN.

In Group 2 (17% EDTA), showed the mean value of hardness of 51.39 ± 1.96 VHN. Statistical analysis showed that there was highly significant difference between Group 1 and Group 2 ($P < 0.0001$). Results obtained within the experimental conditions of the present study indicates that, 17 % EDTA solution leads to structural changes, as evidenced by significant reduction of dentin microhardness compared with the control group.

The effect of EDTA on reducing dentin microhardness has been reported by Poggio et al[41] Ari et al[62] and De-Deus et al[13]. Sayin et al[31] reported that EDTA alone or followed by 2.5% NaOCl promoted a significantly greater decrease in dentin microhardness when compared with EGTA, a calcium-ion– specific chelator, and a combination of EDTA with a tensoactive agent (EDTAC). It is interesting to mention that when the root canal is irrigated with NaOCl followed by EDTA, the collagen degradation with a consequent decrease of flexural strength is caused by a hypochlorite action and has no association with the demineralization promoted by the final rinse with EDTA.[31]

The fact that EDTA acts efficiently in the reduction of dentin microhardness is because of its chelating property. Several theories have tried to explain this chemical reaction. According to the crystalline field theory, the attraction force between the central metal and the ligands is purely electrostatic. Therefore, the attraction force exerted by the metallic ion is greater than the repulsive force offered by the atoms of the EDTA molecule. Chelators such as EDTA form a stable complex with the calcium ions in dentin. In this moment, carboxyl groups of the EDTA molecule are ionized, releasing hydrogen atoms that compete with the calcium ions. This demineralizing effect leads to reduction of microhardness of root dentin.[22]

In Group 3 (15% Citric Acid) has the minimum mean value of hardness of 49.64 ± 1.47VHN. The statistical analysis showed that there was highly significant difference when compared with Group 1 (Distilled water) but there was no significant difference when compared with Group 2 (17% EDTA). The effect of EDTA and citric acid solutions had the strongest effect on reducing dentin microhardness and statistically similar. This results are in accordance with the studies Cruz-Filho et al.[15] who found that 15% EDTA and 10% citric acid promoted greater reduction in microhardness. Both solutions acted similarly, supporting the results of the present study. However, Eldeniz et al[63] reported that 19% citric acid is more efficient in reducing dentin microhardness than 17% EDTA, whereas De Deus et al[13] had opposite results, which differs from our results. The efficiency of chelating agents depends on time of application, pH, concentration of the solution, and amount of solution. Eldeniz et al[63] used 19% citric acid (ie, a higher concentration than that used in the present experiment). In the study by De Deus et al[13] citric acid used was of

lesser concentration (10%). It has been shown that the higher the concentration of a solution, the stronger the chelating effect.[64]

In Group 4, 0.2% chitosan showed mean value of microhardness of 57.06 ± 3.98 VHN. The statistical analysis showed that there was highly significant difference when compared with Group 1 (Distilled water) and Group 3 (15% Citric acid) ($P < 0.0001$) and significant difference when compared with Group 2 (17% EDTA) ($P=0.0005$). In the present study, percentage dentin microhardness reduction was significantly more in the Citric acid and EDTA group ($P < 0.05$) than chitosan. This results are in accordance with the study by Nikhil V et al[65] who concluded that 0.2% Chitosan resulted in significantly lesser microhardness reduction of the radicular dentin than EDTA. Whereas Pimenta et al[36] and Saha SG et al[43] concluded contradictory to the results obtained in present study, that 0.2% chitosan, EDTA, and citric acid reduced root canal microhardness with no statistically significant difference among the solutions.

As described in the Materials and Methods section, chitosan was diluted in 1% acetic acid for preparation of the solution. Thus, it could be speculated that the chelating effect observed in this study was due to the acid and not chitosan. However, previous studies have shown that the capacity of 5% acetic acid for reducing dentin microhardness, and removing the smear layer and chelating calcium ions in the root canal was insignificant in relation to 15% EDTA and 10% citric acid. In this way, it is highly evident that the effect caused by chitosan on dentin microhardness is exclusively due to the substance and not to the acid.[10,36]

The correlation between calcium analysis and microhardness test was obtained and showed that there was a strong negative correlation between calcium loss and hardness i.e. as the percent calcium loss increases, the hardness decreases. This results were in accordance with prior studies done by Thangaraj et al[32] and Taneja S et al[40] who found similar relation between calcium loss and microhardness of root dentin with EDTA, Citric acid and Paracetic acid.

Prior to the clinical use of a new substance or product, further studies are needed to investigate in detail its physical, chemical, and biological properties in order to verify the benefits and consequences to humans. It is essential to conduct further studies to check for the effect of 0.2% chitosan on the surface roughness of the root dentin, adhesion of bacteria and root canal fillings to root dentin, and also the fracture resistance of roots after treatment with 0.2% chitosan solutions at different time intervals.

LIMITATIONS

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1. This was an in vitro study, thus the exact simulation of the oral conditions was not possible. Therefore, the results cannot be directly extrapolated to the clinical situation.

2. Diamond disc was used for sectioning of teeth. Nevertheless, teeth loss in inevitable in sectioning and this could affect the hardness of tooth.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Irrigation of root canal system provides gross debridement, lubrication, destruction of microbes, dissolution of tissues and help in cleaning areas that are inaccessible for mechanical cleansing. During irrigation, radicular and coronal dentin is exposed to irrigating solution deposited in the pulp chamber, which may cause alteration on dentin. The chelating agents have a decalcifying property which results in extracting element like calcium and various oher elements from the dentin causing softening of calcified components of dentin, which could result in reduction in the microhardness of the root canal dentin.

Thus, the present study was undertaken to compare & evaluate the effect of various Chelating Agents on calcium Content and microhardness of root dentin

60 extracted single rooted teeth which fulfilled the inclusion criteria were selected for the study. Teeth were decoronated and split longitudinally and divided into four different groups randomly as follows

Group 1 – Distilled water

Group 2 – 17% EDTA solution

Group 3 – 15% Citric acid solution

Group 4 – 0.2% Chitosan solution

All samples were immersed in their 10 ml of solution depending on the groups for 2 minutes. The samples were then subjected for mechanical testing using ICP – AES technique and Vickers Microhardness testing for calcium analysis and microhardness test. The values was obtained from the software based system attached to the machine.

The results obtained indicated that there is highly significant difference between the mean calcium loss and reduction in microhardness of all four groups (p value <0.0001). This implied that all chelating agents were able to cause calcium loss and reduction of microhardness of root dentin after 2 minutes of exposure. Out of which 15% Citric acid caused highest amount of calcium loss and reduction in microhardness. Whereas 0.2 % Chitosan caused significantly less amount of calcium loss and reduction in microhardness of root dentin when compared to 17% EDTA and 15% Citric acid.

Thus, it can be said that 0.2% Chitosan is much safer than 17% EDTA and 15% Citric acid in terms causing mineral loss and reduction in hardness of dentin.

Within the limitation of present study, the following conclusion can be drawn:

(1) Except Distilled water, all tested chelating agents caused calcium loss and reduced the microhardness of human root dentin.

(2) Despite being structurally different, commonly used chelating agent like 17% EDTA and 15% Citric acid caused more calcium loss and reduction of microhardness of root dentin.

(3) Though 0.2% Chitosan caused calcium loss and reduction in microhardness, there was significant difference when compared with 17% EDTA and 15% Citric acid.

Hence, it may be concluded that a natural derivative chitosan may serve as an effective alternative to the conventionally used root canal irrigants as they cause minimal alteration of dentin structure in addition to being less toxic when compared with synthetic irrigants.

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TABLES AND GRAPHS

Table 1: Descriptive statistics for Calcium loss (%) in four study groups

Groups	N	Mean	SD	Median	Min	Max	95% CI
Control	15	0.29	0.12	0.26	0.12	0.59	0.22 – 0.35
EDTA	15	35.08	22.22	26.30	11.88	79.20	24.08 – 46.08
Citric Acid	15	50.95	18.87	46.26	21.63	81.19	41.45 – 60.45
Chitosan	15	16.20	2.15	16.59	10.40	19.24	15.10 – 17.30

Table 2A: One-way analysis of variance for Calcium loss

Source of Variation	DF*	Sum Squares	Mean Sum Squares	F value	P-value
Between Groups	3	21921	7307	34.20	< 0.0001
Residuals	56	11964	214		

*Degrees of freedom

Table 2B: Pair wise comparison of mean Calcium loss between different groups

Groups	Absolute mean difference	P-value
Control – EDTA	34.786	< 0.0001 (HS)
Control – Citric acid	50.661	< 0.0001 (HS)
Control – Chitosan	15.911	0.0214 (S)
EDTA – Citric acid	15.874	0.0218 (S)
EDTA – Chitosan	18.875	0.0044 (S)
Citric acid – Chitosan	34.749	< 0.0001 (HS)

S: Significant; HS: Highly significant

Table 3: Descriptive statistics for Microhardness in four study groups

Groups	N	Mean	SD	Median	Min	Max	95% CI
Control	15	71.51	5.70	72.42	63.70	81.30	68.61 – 74.41
EDTA	15	51.39	1.96	51.39	48.33	54.10	50.40 – 52.38
Citric Acid	15	49.64	1.47	49.11	47.80	52.06	48.9 – 50.38
Chitosan	15	57.06	3.98	55.50	51.57	64.10	55.06 – 59.06

Table 4A: One-way analysis of variance for Microhardness

Source of Variation	DF*	Sum Squares	Mean Sum Squares	F value	P-value
Between Groups	3	4434	1477.9	108.9	< 0.0001
Residuals	56	760	13.6		

*Degrees of freedom

Table 4B: Pair wise comparison of Microhardness between groups

Groups	Absolute mean difference	P-value
Control – EDTA	20.119	< 0.0001 (HS)
Control – Citric acid	21.873	< 0.0001 (HS)
Control – Chitosan	14.454	< 0.0001 (HS)
EDTA – Citric acid	1.753	0.5647 (NS)
EDTA – Chitosan	5.665	0.0005 (S)
Citric acid – Chitosan	7.418	< 0.0001 (HS)

NS: Not significant; S: Significant; HS: Highly significant

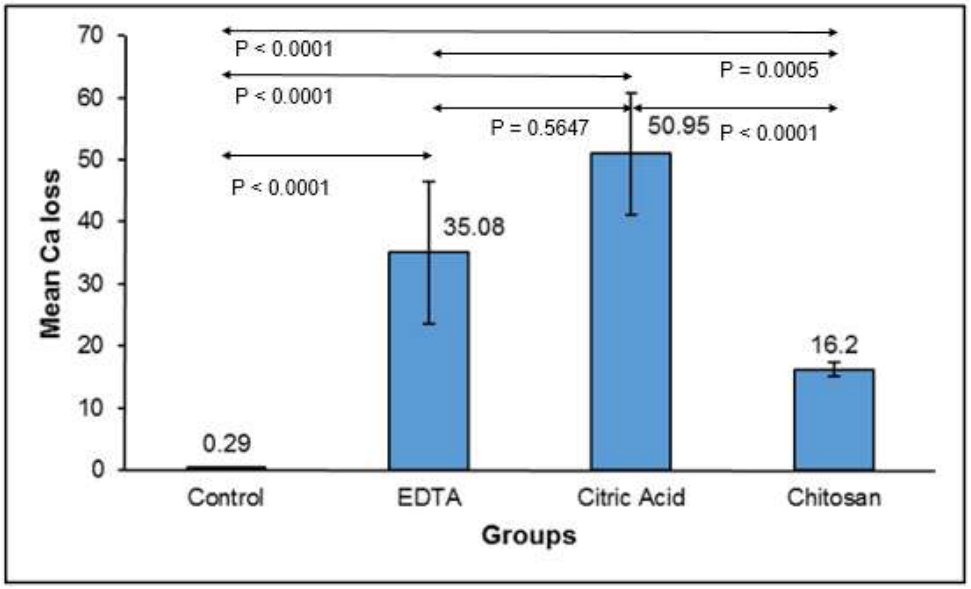

Figure 1: Column chart showing the mean Calcum loss in four study groups

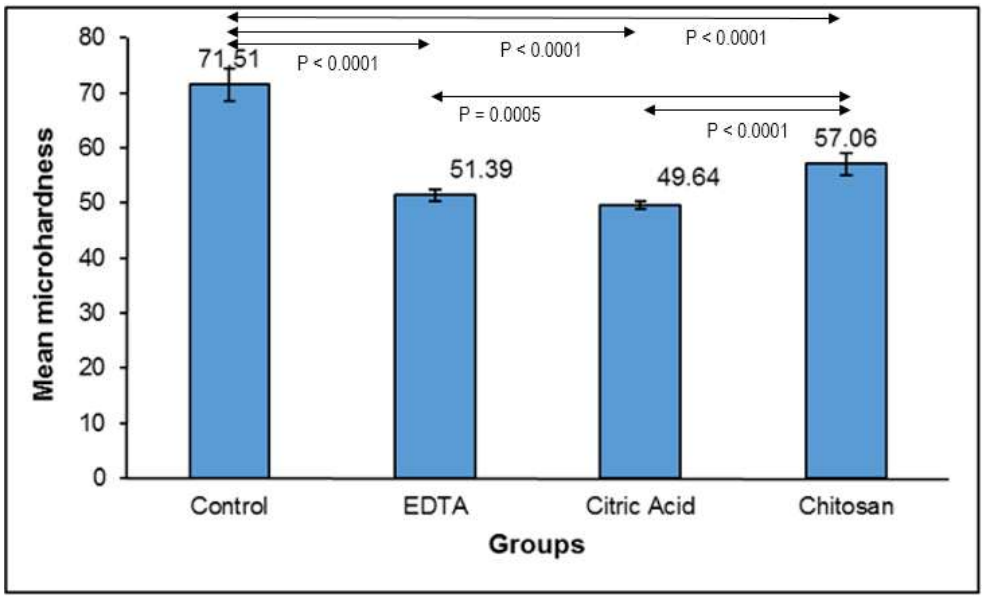

Figure 2: Column chart showing the mean Microhardness in four study groups

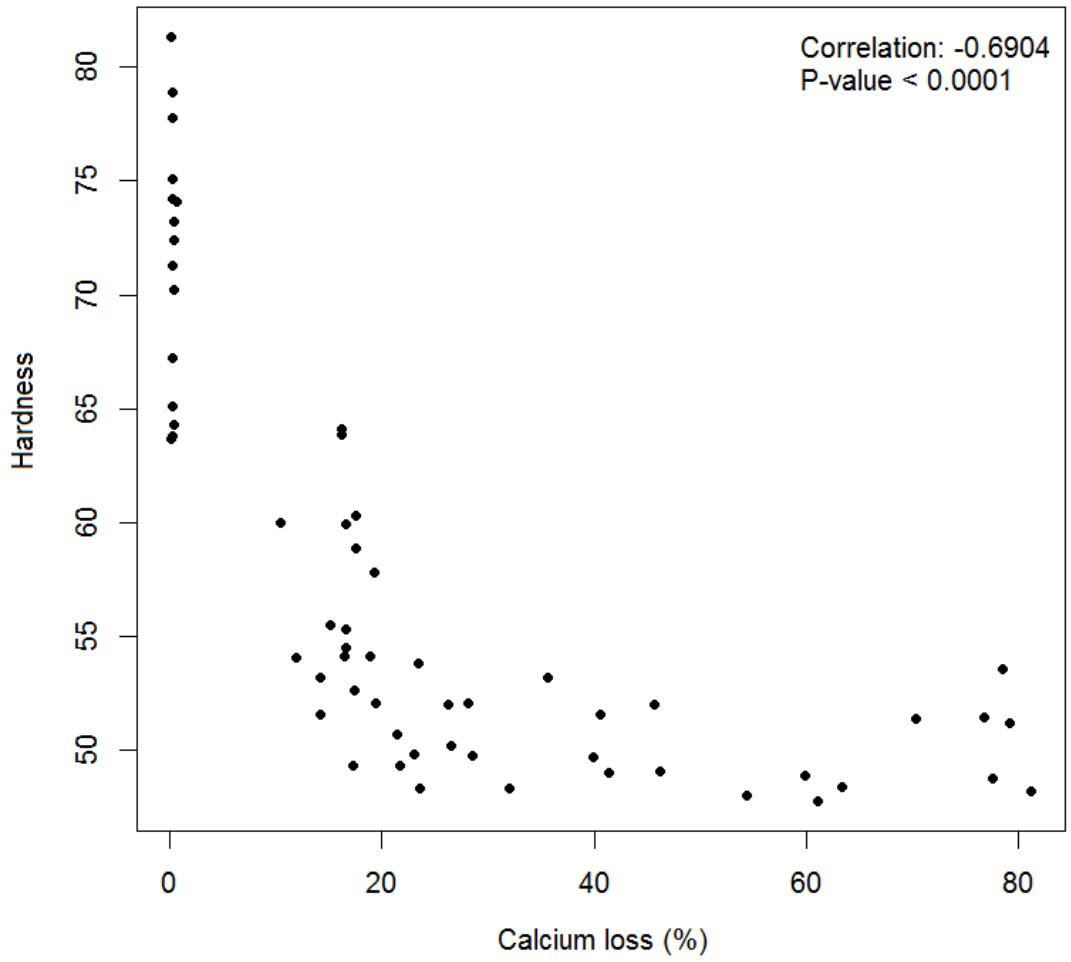

Figure 3: Scatter plot showing correlation of Calcium loss and Microhardness

ANNEXURE

Group 1 : Distilled Water

Calcium loss (In ppm)	
1	0.287
2	0.424
3	0.222
4	0.241
5	0.226
6	0.264
7	0.158
8	0.337
9	0.592
10	0.212
11	0.302
12	0.226
13	0.124
14	0.402
15	0.358

Micro Hardness (in VHN)	
1	77.8
2	64.3
3	65.1
4	71.3
5	74.2
6	78.9
7	81.3
8	70.22
9	74.1
10	63.8
11	75.1
12	67.22
13	63.7
14	73.2
15	72.42

Group 2 : 17 % EDTA

Calcium loss (In ppm)	
1	79.195
2	78.516
3	28.469
4	31.961
5	70.405
6	23.034
7	21.415
8	17.255
9	35.667
10	23.561
11	23.475
12	19.441
13	35.592
14	26.3
15	11.881

Micro Hardness (in VHN)	
1	51.2
2	53.58
3	49.8
4	48.33
5	51.39
6	49.82
7	50.7
8	49.32
9	53.2
10	48.33
11	53.8
12	52.1
13	53.2
14	52
15	54.1

Group 3 : 15 % Citric Acid

Calcium loss (In ppm)	
1	28.172
2	40.525
3	59.92
4	61.042
5	41.385
6	45.711
7	81.188
8	21.629
9	76.741
10	77.55
11	26.472
12	46.256
13	54.42
14	63.424
15	39.853

Micro Hardness (in VHN)	
1	52.06
2	51.57
3	48.9
4	47.8
5	49
6	52.01
7	48.22
8	49.31
9	51.45
10	48.77
11	50.21
12	49.11
13	48.03
14	48.43
15	49.7

Group 4 : 0.2 % Chitosan

Calcium loss (In ppm)	
1	17.469
2	18.2
3	18.888
4	14.164
5	16.586
6	17.566
7	16.429
8	16.583
9	16.246
10	16.608
11	15.078
12	16.203
13	17.353
14	14.235
15	19.238

Micro Hardness (in VHN)	
1	60.3
2	60
3	54.11
4	51.57
5	54.5
6	58.9
7	54.11
8	55.31
9	63.88
10	59.95
11	55.5
12	64.1
13	52.62
14	53.2
15	57.8