Original Article

# Effect of collagen cross-linking agents on the depth of penetration of bioceramic sealer and release of hydroxyproline: An *in vitro* study

K. Hanisha Reddy, Bollineni Swetha[1], B. Devi Priya[1], T Murali Mohan[2], Duvvuri Lakshmi Malini[1], M. Sai Sravya[3]

Associate Professor, [1]Assistant Professor, [2]Professor and HOD, Department of Conservative Dentistry and Endodontics, [3]Government Dental College and Hospital, Vijayawada, Andhra Pradesh, India

## Abstract

**Context:** During endodontic treatment, sealers seal off dentinal tubules and prevent microbial attack. Bioceramic sealers have excellent bioactivity, but its high alkalinity is found to have detrimental effects on radicular collagen. Collagen cross linkers have the ability to chemically modify collagen and can prevent the detrimental effects of the sealer.

**Aim:** This research was aimed to assess the effect of collagen cross-linking agents on the integrity of radicular collagen matrix and depth of penetration of sealer.

**Materials and Methods:** Mandibular premolars ($n$ = 48) were taken. Teeth were decoronated; canals were prepared till ProTaper size F2 and were irrigated with 5 mL of 2.5% NaOCl, followed by 3 mL of 17% ethylenediaminetetraacetic acid between instrumentation and finally rinsed with saline following which teeth were divided into three groups based on the surface treatments: Group 1: 6.5% proanthocyanin (PA), Group 2: chlorhexidine (CHX), and Group 3: saline. Teeth were obturated using gutta-percha and bioceramic sealer and stored in artificial saliva. Hydroxyproline (HYP) release was assessed after 14 and 21 days using spectrophotometer. Sealer penetration was assessed using the scanning electron microscope.

**Statistical Analysis:** Wilcoxon signed-rank test and Kruskal–Wallis test for release of HYP and paired *t*-test and ANOVA for sealer penetration were performed.

**Results:** Significantly lower release of HYP was seen in proanthocyanin-treated group. Sealer penetration was better for both the proanthocyanin- and CHX-treated groups when compared to saline.

**Conclusion:** Surface treatment with collagen cross-linkers caused a decrease in the amount of HYP released, indicating lesser degradation of collagen. Sealer penetration was better due to the removal of smear layer following the surface treatments.

**Keywords:** Bioceramic sealer; collagen; cross-linkers; hydroxyproline; proanthocyanin

## INTRODUCTION

Root canal treatment requires complete chemomechanical debridement of the root canal system following which three-dimensional obturation of the root canal system is to be achieved to provide a proper fluid-tight seal. This is achieved by the use of a sealer along with gutta-percha.[1]

Root canal sealers seal off the root canal system, entombing the remaining bacteria. Various sealers are available, of which bioceramic sealers are most commonly used due to its advantages which include its biocompatibility,

**Address for correspondence:**
Dr. K. Hanisha Reddy,
Department of Conservative Dentistry and Endodontics,
Government Dental College and Hospital, Vijayawada,
Andhra Pradesh, India.
E-mail: sravyamummidivarapu@gmail.com

Date of submission : 09.05.2023
Review completed : 29.09.2023
Date of acceptance : 07.10.2023
Published : 08.02.2024

Access this article online

Quick Response Code:

Website: https://journals.lww.com/jcde

DOI: 10.4103/jcd.jcd_309_23



**How to cite this article:** Reddy KH, Swetha B, Priya BD, Mohan TM, Malini DL, Sravya MS. Effect of collagen cross-linking agents on the depth of penetration of bioceramic sealer and release of hydroxyproline: An *in vitro* study. J Conserv Dent Endod 2024;27:170-4.

antibacterial property, water sorption, and bioactivity. Despite these advantages, these sealers have a limitation of causing high alkalinity, which may have detrimental effects on exposed collagen.[2]

The exposed collagen fibers are fragile and can be easily attacked by endogenous proteases, leading to biodegradation of the dentin eventually leading to fracture of tooth.[3]

Cross-linking has become an important method to slow down the biodegradation rate and enhance the mechanical properties of the collagen. Collagen cross-linking agents which can provide intra and intermolecular cross-links include riboflavin, proanthocyanin, chlorhexidine (CHX), genipin, green tea, epigallocatechin gallate, Biocalein, Quercetin, Naringin, Cardol, and Cardinal.[4]

Proanthocyanin (PAs) are polyphenolic bioflavonoids obtained naturally in plant metabolites. They lead to stable hydrogen bonding and generate nonbiodegradable collagen matrix. They are found to be nontoxic cross-linking agents.[5,6]

The aim of the present study was to assess the effect of collagen cross-linking agents on the integrity of the radicular collagen matrix and the depth of penetration of the sealer.

## MATERIALS AND METHODS

Freshly extracted mandibular premolars ($n$ = 48) with mature apex were taken. Teeth with caries or developmental defects were excluded from the study. The teeth were cleared of tissue debris.

### Preparation of specimen

For the preparation of the tooth samples, the mandibular premolars were decoronated at a length of 12 mm from the apex. Canal preparation of the decoronated premolar was done till F2 ProTaper with the use of 5 mL of 2.5% NaOCl for 1 min between instrumentation and following which 3 mL of 17% ethylenediaminetetraacetic acid was used for 1 min to remove the smear layer. A final rinse was done with the use of saline. 6.5% proanthocyanin was prepared by dissolving 6.5 g of grape seed extract (INLIFE Grape Seed Extract Proanthocyanidins >95%) in 100 mL of distilled water.[7,8]

The samples were randomly divided into three groups based on the surface treatment performed.

- Group 1: 3 mL of 6.5% proanthocyanin for 1 min ($n$ = 16)
- Group 2: 3 mL of 2% CHX for 1 min ($n$ = 16)
- Group 3: Saline for 1 min ($n$ = 16).

The canals were now dried with absorbent paper points and obturated with gutta-percha and a bioceramic sealer (NINETEN NT BIOCERA FLO). The teeth were now stored in artificial saliva at room temperature.

### Assessment of hydroxyproline release

Assessment of the amount of hydroxyproline (HYP) released in the storage medium was performed using HYP assay to determine the solubilized collagen peptides from the dentin cylinder as follows:

Two hundred microliters of vortexed storage medium was gathered from each tube and placed in an individually labeled ampule. The HYP content was analyzed using a spectrophotometer in the transmission mode at 558 nm. The HYP released from solubilized collagen peptide fragments (Sa) was calculated using a regression equation derived from absorbance values obtained from known concentrations of HCl hydrolyzed HYP.[8] [Figure 1].

Sa/Sv = C

Sa = The amount of HYP, calculated from the standard curve (in μg)

Sv = The volume of sample hydrolysate added to the tube (in μL)

C = Concentration of HYP in the sample.

### Assessment of sealer penetration

The samples were stored at 37°C and 100% humidity for 2 weeks. The roots were embedded in resin. Thereafter, the samples were transversely sectioned at 2 and 5 mm from the apex. The samples were dehydrated and mounted on a tub and gold-sputtered. The specimens were observed in a scanning electron microscope (SEM) at 15 kV accelerating voltage with ×2500 magnification.[9]

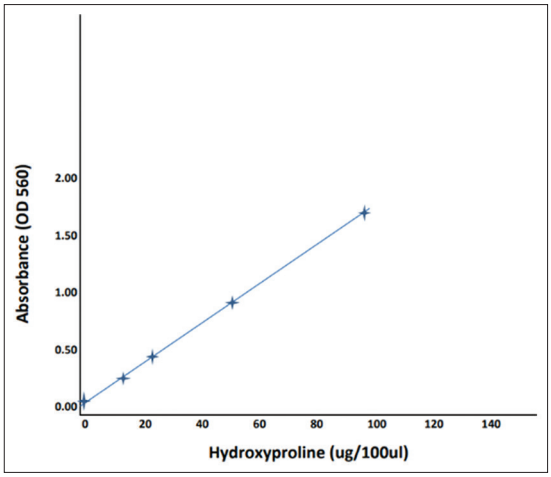



**Figure 1:** Hydroxyproline standard curve

### Statistical analysis

The amount of HYP release and the amount of sealer penetration were tabulated and statistically analyzed using SPSS (Statistical Packages for the Social Sciences, version 21.0., IBM Corp., Armonk, NY, USA). The amount of HYP released was analyzed using Wilcoxon signed-rank and Kruskal–Wallis test. The sealer penetration depths were assessed using paired *t*-test and ANOVA test. The level of significance was set at $P = 0.05$.

## RESULTS

### Release of hydroxyproline

The mean amount of HYP released was least in the proanthocyanin-treated group both after 14 and 21 days (275.166 Ug and 297.666 Ug, respectively) followed by CHX [Table 1 and Supplementary Figure 1].

### Sealer penetration

Proanthocyanin and CHX groups did not exhibit any statistically significant differences in the quantity of sealer penetration, according to *post hoc* test [Supplementary Table 1].

The amount of sealer penetration for proanthocyanin- and CHX-treated group was found to be highest both in the middle and apical third [Table 2, Figure 2 and Supplementary Figure 2].

## DISCUSSION

The use of bioceramic materials as sealers has two main benefits, their biocompatibility and the presence of calcium and phosphate, which improves their setting properties and results in a chemical composition and crystalline structure similar to materials made of tooth and bone apatite, improving the bonding of the sealer to the root dentin.[10]

Despite the bioactivity of bioceramic sealants, the high alkalinity of these substances may harm the collagen matrix of the dentin. They weaken the intermolecular connections between collagen fibrils, causing the interfacial dentin's collagenous component to deteriorate and its permeability to increase.[11]

According to Yang *et al*., the degradation of collagen fibrils can reduce the flexural strength of dentin, its microhardness,

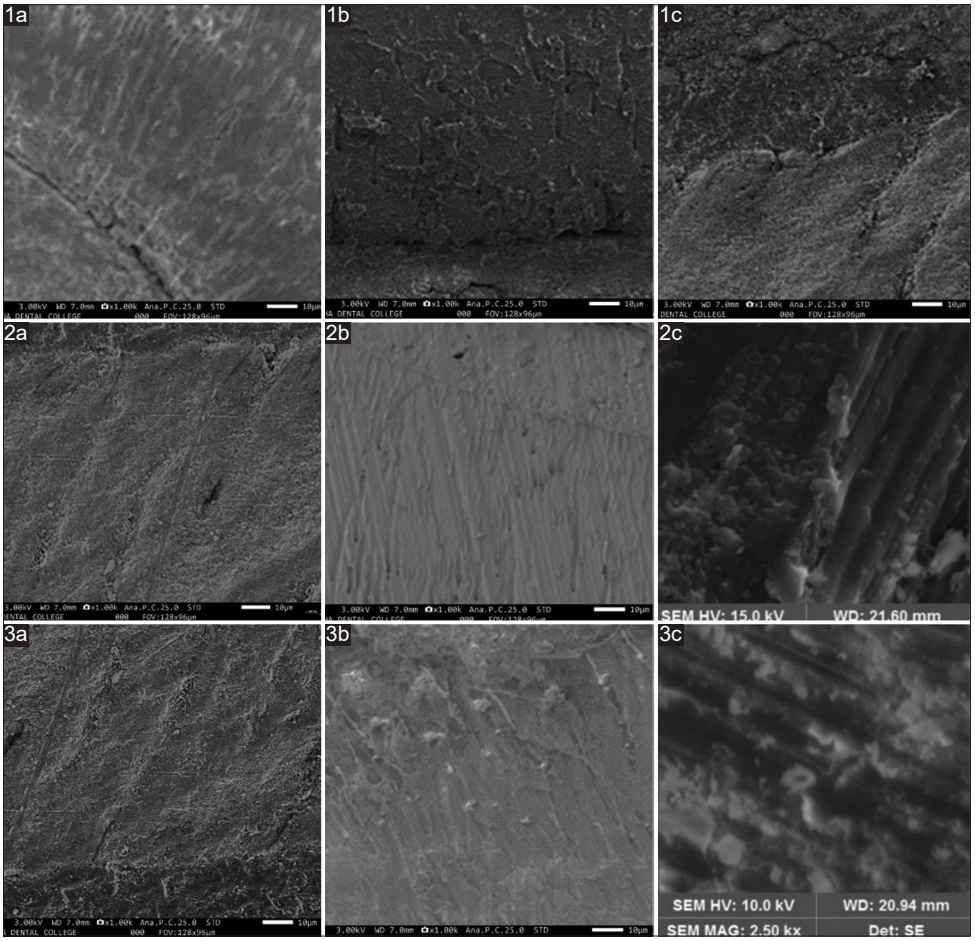



**Figure 2:** Sealer penetration at a magnification of ×2500 (1a) at coronal third, (1b) middle third, and (1c) apical third for proanthocyanin-treated groups (2a) at coronal, (2b) middle, and (2c) apical third for chlorhexidine-treated groups. (3a) at coronal, (3b) middle, and (3c) at apical third of saline-treated groups

**Table 1: Comparison of hydroxyproline release among the groups**

| Group | Timeline (days) | *n* | Mean | SD | Mean difference | Test statistic | *P* |
|---|---|---|---|---|---|---|---|
| PA | 14 | 8 | 275.1667 | 21.36742 | −22.500 | −1.572 | 0.116 |
| | 21 | 8 | 297.6667 | 13.36663 | | | |
| CHX | 14 | 8 | 351.8333 | 9.43221 | −30.166 | −1.992 | 0.046* |
| | 21 | 8 | 382.0000 | 16.43168 | | | |
| Saline | 14 | 8 | 963.3333 | 7.84007 | −35.000 | −1.992 | 0.046* |
| | 21 | 8 | 998.3333 | 28.84903 | | | |

CHX: Chlorhexidine, SD: Standard deviation, PA: Proanthocyanin, *$P<0.05$ significant in group 2 and group 3 showing least amount of hydroxyproline release in group 1

**Table 2: Comparison of sealer penetration among the groups**

| Group | Timeline | *n* | Mean | SD | Mean difference | *t* | *P* |
|---|---|---|---|---|---|---|---|
| PA | Middle | 8 | 36.0000 | 4.51664 | 19.1667 | 8.548 | 0.000* |
| | Apical | 8 | 16.8333 | 1.94079 | | | |
| CHX | Middle | 8 | 42.8333 | 4.44597 | 20.333 | 8.661 | 0.000* |
| | Apical | 8 | 23.5000 | 4.08656 | | | |
| Saline | Middle | 8 | 24.6667 | 4.27395 | 11.500 | 6.252 | 0.002* |
| | Apical | 8 | 13.1667 | 2.78687 | | | |

CHX: Chlorhexidine, SD: Standard deviation, PA: Proanthocyanin, *$P<0.05$ more significant in groups 1 and 2 showing better penetration of sealer compared to saline

and the resistance of the root to fracture.[12] Type I collagen contributes to most of the radicular dentin, of which 9.6% of collagen is constituted by HYP. Moreover, HYP stabilizes the triple helical structure of collagen. Therefore, the amount of HYP released can serve as a marker for assessing the degradation of collagen.[13,14]

According to Mishra *et al*., the surface treatment can directly interfere with dentin permeability and quality of bond strength of the root canal filling materials, consequently impacting the success of endodontic therapy.[15]

Pretreatment of radicular dentin collagen with exogenous collagen cross-linking agents, before the application of sealer might result in mechanical and biological stabilization of collagen.[16]

Proanthocyanins cause the dentin proteases sites into inactive mode by lowering their molecular mobility, causing conformational changes to their structure, or by converting their negatively charged ionized carboxyl groups to positively charged amides which are responsible for collagen degradation and they also cause stable inter- and intramolecular cross-links in the collagen that hampers degradation.[3,17]

Proanthocyanin was used for surface pretreatment in the current study, as it is nontoxic and possesses excellent biocompatibility, antibacterial, and antioxidant properties. Proanthocyanins are known to inhibit bacterial proteases and host-derived matrix metalloproteinases. It can also enable collagen biosynthesis by binding to proline-rich proteins, such as collagen, and facilitates the activity of enzyme proline hydroxylase that can result in achieving long-term collagen stabilization.[18]

2.5% NaOCl was used in this study. The use of higher concentrations of NaOCl can result in dissolution of organic matter and can induce the degradation of dentinal collagen through the breakdown of carbon atom bonds and disorganization of protein structure, resulting in dentin degeneration.[19]

HYP release can be estimated by chromatographic method or spectroscopic method. Spectrophotometric determination of HYP is adequately reproducible.[20] Therefore, spectrophotometer was used in this study for the estimation of HYP.

In our study, the release of HYP was assessed on 14$^{th}$ and 21$^{st}$ day. This is because, there is an initial high release of HYP which is followed by stable release of HYP.[21]

In the present study, the amount of HYP released is least in proanthocyanin-treated group, which indicates that the amount of collagen degradation is least. This is explained by the fact that proanthocyanin serves as a collagen cross-linker, stimulating the interfibrillar, intrafibrillar, and intermicrofibrillar cross-links in the collagen and protecting it from degradation by exogenous collagenase. This improves the biomechanical characteristics and biostability of dentin collagen.[22]

The amount of HYP release was found to be less in CHX-treated group. This may be owing to CHX's capacity to cross-link collagen, which may be brought by its cationic characteristics and strong affinity for both organic (hydroxyapatite) and inorganic (collagen) dentin structures.[23]

Sealer penetration depths were assessed in this study. This is because sealer penetration depths and their interfacial adaptations are important factors in the determination of the success of root canal treatment. SEM was used as the defects at the submicron level are often observed at high magnification.[24]

According to the present study, sealer penetration was better for proanthocyanin- and CHX-treated groups compared to saline. This can be attributed to the fact that proanthocyanin has the capacity to dissolve the smear layer and CHX-containing surfactant in its composition,

increases the surface energy, wettability, and enhances the cationic properties of dentin, thereby making sealer penetration better.[25,26]

Penetration of sealer was found to be lesser in the apical region compared to middle and coronal. This could be due to decreased tubular density, presence of sclerosed dentin, reduced effectiveness of irrigant in the apical region, thereby reduced smear layer removal.[27,28]

The limitation of the present study was the preparation method used for preparing SEM specimens. There is a risk of tearing or smearing of GP and sealer while sectioning of the filled root. Slight discoloration was observed in specimens treated with proanthocyanin. Further studies need to be conducted to know its role as a when used *in vivo*.

## CONCLUSION

The alkalinity of the bioceramic sealer is one of the many causes of root fracture following obturation, but it is also one of the least understood. Collagen degradation can therefore be stopped using collagen cross-linkers, which will also stop the root fracture from happening.

Within the limitations of this study, surface treatment with proanthocyanin and CHX following irrigation caused a decrease in the amount of HYP released, which indicates that there is lesser degradation of collagen in the root dentin. Sealer penetration was better due to the removal of smear layer for the surface treatments groups.

Hence, the usage of cross-linkers can be implied in our root canal procedures before obturation so as to prevent the root degradation changes which eventually lead to fracture of tooth which were caused by the bioceramic sealer and also for better sealer penetration.

### Financial support and sponsorship
Nil.

### Conflicts of interest
There are no conflicts of interest.

## REFERENCES

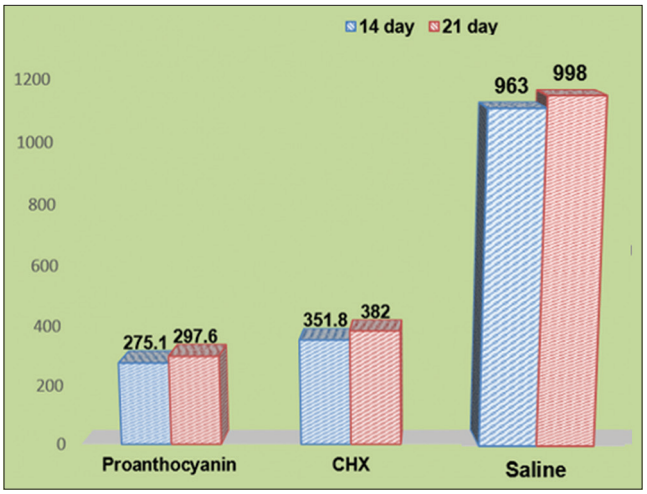
1. Komabayashi T, Colmenar D, Cvach N, Bhat A, Primus C, Imai Y. Comprehensive review of current endodontic sealers. Dent Mater J 2020;39:703-20.
2. Huang XQ, Camba J, Gu LS, Bergeron BE, Ricucci D, Pashley DH, *et al.* Mechanism of bioactive molecular extraction from mineralized dentin by calcium hydroxide and tricalcium silicate cement. Dent Mater 2018;34:317-30.
3. Hardan L, Daood U, Bourgi R, Cuevas-Suárez CE, Devoto W, Zarow M, *et al.* Effect of collagen crosslinkers on dentin bond strength of adhesive systems: A systematic review and meta-analysis. Cells 2022;11:2417.
4. Sapuła P, Bialik-Wąs K, Malarz K. Are natural compounds a promising alternative to synthetic cross-linking agents in the preparation of hydrogels? Pharmaceutics 2023;15:253.
5. Han B, Jaurequi J, Tang BW, Nimni ME. Proanthocyanidin: A natural crosslinking reagent for stabilizing collagen matrices. J Biomed Mater Res A 2003;65:118-24.
6. Srinivasulu S, Vidhya S, Sujatha M, Mahalaxmi S. Effect of collagen cross-linkers on the shear bond strength of a self-etch adhesive system to deep dentin. J Conserv Dent 2013;16:135-8.
7. Navjot SM, Ashu J, Kamalpreet K, Navneet KM, Manu R, Divya B. The effect of natural reducing agents on push-out bond strength of AH plus and BioRoot RCS to sodium hypochlorite treated root dentin. J Conserv Dent 2021;24:130-4.
8. Ismail SM, Fayyad DM, Eldaharawy MH, Mohamed DA. Effect of two calcium-silicate sealers and a resin sealer on collagen matrix integrity of root dentin after different treatments. An *in vitro* and *in vivo* study. Saudi Endod J 2022;12:67-71.
9. Chen H, Zhao X, Qiu Y, Xu D, Cui L, Wu B. The tubular penetration depth and adaption of four sealers: a scanning electron microscopic study. BioMed Research International 2017.
10. Al-Haddad A, Che Ab Aziz ZA. Bioceramic-based root canal sealers: A review. Int J Biomater 2016.
11. Leiendecker AP, Qi YP, Sawyer AN, Niu LN, Agee KA, Loushine RJ, *et al.* Effects of calcium silicate-based materials on collagen matrix integrity of mineralized dentin. J Endod 2012;38:829-33.
12. Yang SY, Liu Y, Mao J, Wu YB, Deng YL, Qi SC, *et al.* The antibiofilm and collagen-stabilizing effects of proanthocyanidin as an auxiliary endodontic irrigant. Int Endod J 2020;53:824-33.
13. Kato MT, Leite AL, Hannas AR, Calabria MP, Magalhães AC, Pereira JC, *et al.* Impact of protease inhibitors on dentin matrix degradation by collagenase. J Dent Res 2012;91:1119-23.
14. Jain K, Beri L, Kunjir K, Borse N, Neekhara N, Kadam A. Comparative evaluation of the effect of 10% sodium ascorbate, 10% hesperidin, 1% riboflavin 5 phosphate, collagen cross-linkers, on the pushout bond strength of fiber postluted to radicular dentin: *In vitro* study. J Conserv Dent 2018;21:95-9.
15. Mishra L, Khan AS, Velo MM, Panda S, Zavattini A, Rizzante FA, *et al.* Effects of surface treatments of glass fiber-reinforced post on bond strength to root dentine: A systematic review. Materials (Basel) 2020;13:1967.
16. Al-Ammar A, Drummond JL, Bedran-Russo AK. The use of collagen cross-linking agents to enhance dentin bond strength. J Biomed Mater Res B Appl Biomater 2009;91:419-24.
17. Sabatini C, Pashley DH. Mechanisms regulating the degradation of dentin matrices by endogenous dentin proteases and their role in dental adhesion. A review. Am J Dent 2014;27:203-14.
18. Arora S, Gordon J, Hook M. Collagen binding proteins of gram-positive pathogens. Front Microbiol 2021;12:628798.
19. Zhang K, Kim YK, Cadenaro M, Bryan TE, Sidow SJ, Loushine RJ, *et al.* Effects of different exposure times and concentrations of sodium hypochlorite/ethylenediaminetetraacetic acid on the structural integrity of mineralized dentin. J Endod 2010;36:105-9.
20. Ignat'eva NY, Danilov NA, Averkiev SV, Obrezkova MV, Lunin VV, Sobol' EN. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. J Anal Chem 2007;62:51-7.
21. Inagati CM, Scheffel DL, Anovazzi G, Alonso JR, Christoffoli MT, Pashley DH, *et al.* Proteolytic activity and degradation of bovine versus human dentin matrices. J Appl Oral Sci 2021;29:e20210290.
22. Bedran-Russo AK, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. J Biomed Mater Res B Appl Biomater 2008;86:330-4.
23. Münchow EA, Bottino MC. Recent advances in adhesive bonding – The role of biomolecules, nanocompounds, and bonding strategies in enhancing resin bonding to dental substrates. Curr Oral Health Rep 2017;4:215-27.
24. Majumdar TK, Mukherjee S, Mazumdar P. Microscopic evaluation of sealer penetration and interfacial adaptation of three different endodontic sealers: An *in vitro* study. J Conserv Dent 2021;24:435-9.
25. Abu Zeid ST, Bastawy HA, Mokeem Saleh AA. Natural extracts as biological smear layer removing agents: A literature review. J Int Soc Prev Community Dent 2021;11:589-600.
26. Al-Zaka IM, Atta-Allah AA, Al-Gharrawi HA, Mehdi JA. The effect of different root canal irrigants on the sealing ability of bioceramic sealer. Mustansiria Dent J 2013;10:1-7.
27. Sreedev CP, Raju I, Kumaravadivel K, Mathew S, Thangavel B, Natesan Thangaraj D. Influence of different types of root canal irrigation regimen on resin-based sealer penetration and pushout bond strength. Cureus 2020;12:e7807.
28. Mokashi P, Shah J, Chandrasekhar P, Kulkarni GP, Podar R, Singh S. Comparison of the penetration depth of five root canal sealers: A confocal laser scanning microscopic study. J Conserv Dent 2021;24:199-203.

## SUPPLEMENTARY FILES

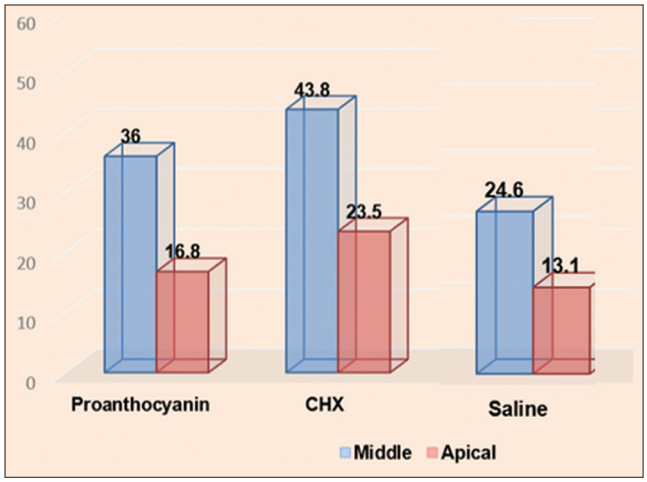
**Supplementary Table 1:** *Post hoc* analysis for sealer penetration

| | *Post hoc* analysis | |
|---|---|---|
| Comparison between | Mean difference | *P* |
| PA | | |
| CHX | −6.833 | 0.083 |
| Saline | 11.333 | 0.001* |
| CHX | | |
| Saline | 19.166 | 0.000* |

CHX: Chlorhexidine, PA: Proanthocyanin




**Supplementary Figure 1:** Hydroxyproline levels at different times between the groups. CHX: Chlorhexidine




**Supplementary Figure 2:** Comparison of penetration depth at different levels between the groups. CHX: Chlorhexidine