Original Article

Biomimetic remineralization of eroded dentin by synergistic effect of calcium phosphate and plant-based biomodifying agents: An *in vitro* study

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Abstract

Context: Dentin, due to its organic content, exhibits a high susceptibility to dental erosion owing to its high permeability to intrinsic acids. A crucial challenge for successful remineralization of demineralized dentin is reestablishing a complete spatial relationship between the mineral and collagen.

Aims: This study aimed to evaluate the synergistic effect of casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACPF) with plant-based dentin biomodifying agents: Grape seed extract (GSE), green tea extract (GTE), and cranberry extract (CE) on biomimetic remineralization of eroded dentin.

Settings and Design: Dentin samples were prepared and subjected to surface microhardness (SMH) analysis at baseline followed by erosive challenge with hydrochloric acid (0.01M) and pH cycling with citric acid (0.05M) and artificial saliva. The samples in all the groups except the control were subjected to surface treatments: CPP-ACPF, GSE + CPP-ACPF, GTE + CPP-ACPF, and CE + CPP-ACPF.

Subjects and Methods: Samples from all the groups were subjected to Vickers Hardness Tester for Post Treatment SMH analysis. Samples were qualitatively evaluated for changes in ultramorphological characteristics of dentin surface using scanning electron microscopy (SEM) and were quantitatively analyzed for changes in the calcium (Ca)/phosphorous (P) ratio using energy dispersive X-ray analysis (EDAX).

Statistical Analysis Used: Data obtained were statistically analyzed using ANOVA, paired samples test, and Tukey HSD test. *P* value < 0.05 was considered statistically significant.

Results: The samples pretreated with GSE showed the highest mean SMH followed by GTE and CE. The difference in % change was significant when GSE was compared to GTE and CE ($P = 0.001$ and $P = 0.000$, respectively). SEM pictomicrographs of demineralized dentin samples revealed enlarged and exposed dentinal tubules, and most of these tubules were unobstructed with no obvious sediments. Samples treated with test agents demonstrated amorphous crystal-like deposits occluding the dentinal tubules with higher incidence observed in the groups treated with the combination of the agents. EDAX analysis revealed the highest mean wt% of Ca and *P* in groups pretreated with GSE followed by GTE and CE. GSE showed the highest mean Ca/P ratio followed by CE and GTE.

Conclusions: The findings of this study demonstrated a synergistic effect when CPP-ACPF was used after pretreatment with plant-based dentin biomodifying agents in causing biomimetic remineralization of eroded dentin.

Keywords: Biomimetic remineralization; collagen stabilization; casein phosphopeptide-ACPF; cranberry extract; dentin biomodification; grape seed extract; green tea extract

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INTRODUCTION

Dental erosion has been prevalent in recent years as a result of dietary and lifestyle changes. Dental erosion is the dissolution of teeth by acids when the surrounding aqueous phase is undersaturated with minerals as compared to the teeth. Erosive damage because of intrinsic acids presents a substantial difficulty in the management of the condition when compared to dental erosion caused by dietary acids.[1]

Fluoride has a primarily inorganic action and its effectiveness in remineralizing eroded enamel cannot be extrapolated in relation to dentin. A crucial challenge for the successful remineralization of dentin is reestablishing a complete spatial relationship between the mineral and the scaffold-collagen. Collagen is essential to modulate the arrangement and growth of the apatite. Collagen's charged groups act as sites for the nucleation of apatite.[2]

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a combination of a bovine milk protein (CPP) complexed with ACP to form nanocomplexes. Through the preservation of a supersaturated condition of calcium and phosphate, it facilitates the remineralization of the erosive lesions.[3] Fluoride has a synergistic effect with CPP-ACP and increases its remineralizing potential through the formation of stabilized amorphous calcium fluoride phosphate. Although CPP-ACPF has demonstrated the ability to remineralize carious and erosive lesions on enamel, its exclusively inorganic action poses a challenge to managing the erosive demineralization of dentin.

Dentin biomodification refers to a biomimetic approach wherein, by modifying the biochemistry and biomechanical properties of dentin using both natural and synthetic sources mediated by bioactive agents, it can be enhanced and reinforced.[4] Although the polyphenols found in plant extracts such as green tea extract (GTE), grape seed extract (GSE), and cranberry extract (CE) have been shown to have dentin biomodifying properties,[5] it is unclear how these extracts might be used to prevent dental erosion.

Hence, this study is designed to evaluate the possible synergistic effect of a calcium phosphate-based agent (CPP-ACPF) used after pretreatment with plant-based dentin biomodifying agents – GSE, GTE, and CE in managing erosive demineralization of dentin. The null hypothesis stated that there is no significant difference between the effectiveness of CPP-ACPF and CPP-ACPF used after pretreatment with plant-based dentin biomodifying agents in managing erosive demineralization of dentin.

SUBJECTS AND METHODS

This *in vitro* was conducted in accordance with the guidelines laid down in the Declaration of Helsinki (2000) and was approved by the Institutional Ethics Committee (ETHICS/ABSMIDS/239/2022). With an estimated 80% power of the test, 1.56% standard deviation, 2.5% margin of error, and 95% confidence level, the sample size was calculated to be 50 ($n = 10$ per group).

Sample preparation

Twenty-five human intact maxillary and mandibular premolars extracted for orthodontic reasons from patients between the age group of 15–30 years were collected, cleaned, disinfected, and handled as per the guidelines laid down by the occupational Safety and Health Administration and screened for defects.

After surface cleansing with an ultrasonic scaler, the enamel was removed to expose the dentin surface. Each tooth was sectioned to obtain 50 dentin blocks of 4 mm × 4 mm × 2 mm each which were finished to prepare a flat surface using fine grit diamond points, polished with silicon carbide abrasive paper (600–1400 grit) under water cooling and mounted in acrylic blocks for surface microhardness (SMH) analysis.[6]

Baseline surface microhardness analysis

The samples were subjected to SMH at baseline using a Vickers Hardness tester (HM-200, Mitutoyo America Corporation, Illinois, USA). Three indentations were made using a 300 g load with a dwell time of 20s and the average of three readings was considered the baseline SMH value.[7]

Erosive challenge

The samples were subjected to erosive challenge 4 times/day for 5 days by immersion into hydrochloric acid (0.01M) for 2 min followed by a wash with deionized water for 20 s and immersion into artificial saliva for 2 h. At the end of each day, the samples were stored in artificial saliva overnight (14 h).

Remineralization regimen

The samples in G1 ($n = 10$) (no treatment) were stored in artificial saliva, whereas samples in G2 ($n = 10$) were subjected to CPP-ACPF for 4 min twice daily for 28 days. G3, G4, and G5 ($n = 10$/group) were pretreated with 6.5% GSE, 6.1% GTE, and 10% CE, respectively, for 60 seconds,[8] followed by CPP-ACPF for 4 min twice daily for 28 days.[9] The samples were rinsed with deionized water after each application and then stored in artificial saliva. All samples were subjected to pH cycling using citric acid (0.05M) and artificial saliva during the study period.

Posttreatment surface microhardness analysis

The samples were again subjected to Vickers Hardness Tester for post-treatment SMH analysis using 300 g load with a dwell time of 15s.

Scanning electron microscopy and energy dispersive X-ray analysis

The samples were dehydrated at 37°C for 48 h in a vacuum chamber following which, they were sputter coated with two layers (≈10 nm) of gold using a metallizer. Operating at 15 kV, the scanning electron microscopy (SEM) (Zeiss EVO MA 18) generated pictomicrographs with a magnification of ×5000 that were qualitatively evaluated for changes in ultramorphological characteristics of dentin surface.

Energy dispersive X-ray analysis (EDAX) (Oxford EDS X-Act) was used to obtain the wt% of calcium (Ca) and phosphorous (P) elements present in the dentin surface which was quantitatively analyzed for changes in the Ca/P ratio.

Statistical analysis

Data obtained were statistically analyzed for the difference in baseline and posttreatment SMH values between various groups using ANOVA and Paired samples test. The difference in posttreatment SMH, % change in SMH, and wt% of Ca and P among various groups was compared and analyzed using the Tukey HSD test. Analysis was done using IBM SPSS Version 23.0 software (Chicago, Illinois, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Surface microhardness analysis

The difference between the mean SMH values at baseline was not statistically significant among the groups ($P = 0.525$). The mean SMH posttreatment was highest for G3 (86.86 ± 5.94) followed by G4 (86.18 ± 4.85), G2 (83.33 ± 5.39), G5 (83.28 ± 4.75), and G1 (52.59 ± 2.91) and the difference between them was statistically significant ($P = 0.000$). The difference between the SMH values at baseline and post-treatment in all 5 groups was statistically significant ($P = 0.000$) [Figure 1a].

The mean difference and the % change in posttreatment SMH values between G1 and the other groups were statistically significant ($P = 0.000$ and $P = 0.000$). There was a significant difference in the % change in posttreatment SMH values between G2-G3, G2-G4, G3-G4, and G3-G5 ($P = 0.000$, $P = 0.006$, $P = 0.001$, and $P = 0.000$, respectively) [Figure 1b and Table 1].

Scanning electron microscopy

SEM pictomicrographs of G1 showed enlarged, exposed, and unobstructed dentinal tubules with no obvious sediments [Figure 2a]. The intertubular dentin was partially demineralized, with an exposed peritubular dentin. Samples from G2 revealed a thin homogenous layer of amorphous crystal-like deposits coating the dentin surface and occluding the dentinal tubules, thereby reducing their diameter [Figure 2b]. SEM pictomicrographs of G3, G4, and G5 showed higher incidences of dentinal tubule occlusion by ACP deposits [Figure 2c-e].

Energy dispersive X-ray analysis

EDAX analysis revealed that the Ca/P ratio was highest in G3 (1.89 ± 0.03), followed by G5 (1.86 ± 0.12), G4 (1.84 ± 0.01), G2 (1.82 ± 0.09), and G1 (1.73 ± 0.13) [Figure 1c]. The mean difference in the Ca/P ratio between G1 when compared to G3 and G5 was statistically significant ($P = 0.004$ and $P = 0.021$, respectively) [Figure 1d and Table 2].

DISCUSSION

The present *in-vitro* study aimed to evaluate the possible synergistic remineralizing effect of CPP-ACPF on Eroded Dentin surface when used after pre-treatment with GSE, GTE, and CE.

In the SMH analysis, G1 showed a significant decrease in mean SMH values compared to the baseline ($P = 0.000$) [Figure 1a], which may be attributed to high acidity of HCl with low pKa value of 6.3 that causes demineralization by the dissociated hydrogen ions mechanism. All groups treated with CPP-ACPF (G2, G3, G4, and G5) showed an increase in mean SMH values and the difference in the % change recorded was significantly higher in these samples when compared to the G1 ($P = 0.000$) [Figure 1b and Table 1] which can be attributed to the remineralizing effect of CPP-ACPF.[6]

The mean SMH value was lower in G2 when compared to the pre-treatment groups G3 and G4 [Figure 1a] and the difference in % change between them was statistically significant ($P = 0.000$ and $P = 0.006$, respectively) [Figure 1b and Table 1]. These results agree with previous literature[10] and may be attributed to the dentin collagen bio-modification effect by the proanthocyanidin (PA) present in GSE and GTE.

PAs represent a category of phenolic compounds occurring universally in woody and certain herbaceous plants that fall under the condensed tannins category. They stabilize the dentin collagen by cross-linking action owing to their covalent, hydrogen bonding, ionic, and hydrophobic interactions with collagen.[11] PA's can displace water between collagen microfibrils causing their aggregation, creating new hydrogen bonds, and thus producing a denser collagen matrix.[12] The biological stability of collagen is also enhanced by PA's nonspecific MMP inhibiting effect through conformational changes in the three-dimensional structure of the enzyme.[13] According to Castellan *et al*., demineralized dentin demonstrated an increased stiffness after being subjected to PA and reported that

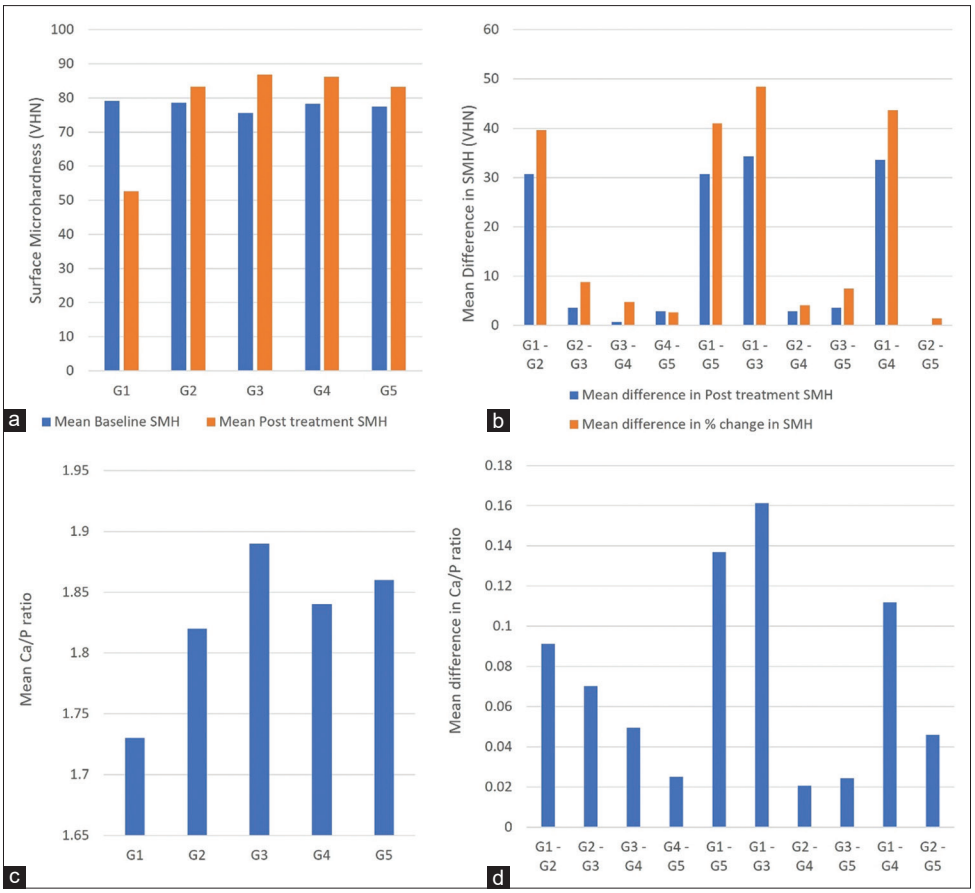

Figure 1a

Group	Mean baseline SMH	Mean posttreatment SMH
G1	79.13	52.59
G2	78.55	83.33
G3	75.65	86.86
G4	78.25	86.18
G5	77.46	83.28

Figure 1b

Groups	Mean difference in posttreatment SMH	Mean difference in (%) change in SMH
G1–G2	30.74	39.6
G2–G3	3.53	8.85
G3–G4	0.68	4.79
G4–G5	2.9	2.62
G1–G5	30.69	41.03
G1–G3	34.27	48.45
G2–G4	2.85	4.06
G3–G5	3.58	7.41
G1–G4	33.59	43.66
G2–G5	0.05	1.43

Figure 1c

Groups	Mean Ca/P ratio
G1	1.73
G2	1.82
G3	1.89
G4	1.84
G5	1.86

Figure 1d

Groups	Mean difference in Ca/P ratio
G1–G2	0.09122
G2–G3	0.07015
G3–G4	0.04946
G4–G5	0.02514
G1–G5	0.13706
G1–G3	0.16138
G2–G4	0.02069
G3–G5	0.02432
G1–G4	0.11192
G2–G5	0.04583

Figure 1: (a) Comparison of baseline and posttreatment surface microhardness (SMH) values among various groups, (b) Comparison of mean difference in posttreatment SMH and mean difference in % change in SMH among various groups, (c) comparison of mean Ca/P ratio between groups, (d) comparison of mean difference in Ca/P ratio between groups. SMH: Surface microhardness

the improvement in the properties of dentin was highly dependent on the source of the PA.[10]

GSE obtained from grape seeds (Vitis vinifera) contains high percentages of PA that strengthen collagen tissues by cross-linking effect.[13] Research has demonstrated the remineralization potential of GSE with gallic acid being one of its major constituents that facilitates mineral deposition.[14] GTE exhibits a high concentration of epigallocatechin-3-gallate which has an inhibitory activity

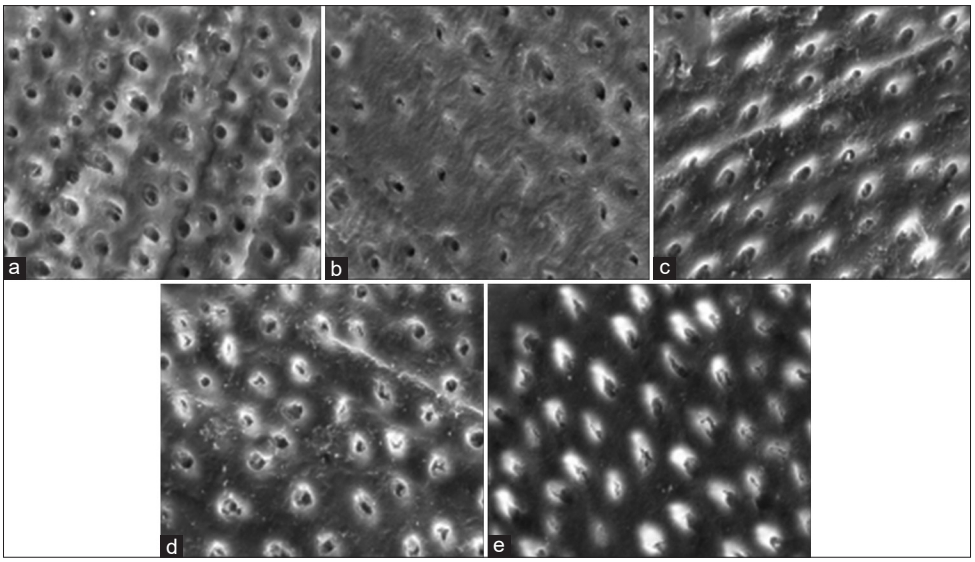

Figure 2: Scanning electron microscope pictomicrographs (×5000): (a) Control-demineralized group, (b) casein phosphopeptide (CPP)-ACPF, (c) grape seed extract + CPP-ACPF, (d) green tea extract + CPP-ACPF, (e) cranberry extract + CPP-ACPF

Table 1: Comparison of mean difference in posttreatment surface microhardness and mean difference in % change in surface microhardness among various groups

Comparison groups	Mean difference posttreatment SMH	95% CI		*P* (Tukey HSD)	Mean difference % change in SMH	95% CI		*P* (Tukey HSD)
		Lower	Upper			Lower	Upper	
G1–G2	−30.74	−36.94	−24.53	0.000*	−39.60	−42.76	−36.43	0.000*
G1–G3	−34.27	−40.47	−28.06	0.000*	−48.45	−51.62	−45.29	0.000*
G1–G4	−33.59	−39.79	−27.38	0.000*	−43.66	−46.82	−40.49	0.000*
G1–G5	−30.69	−36.89	−24.48	0.000*	−41.03	−44.20	−37.87	0.000*
G2–G3	−3.53	−9.73	2.67	0.494	−8.85	−12.02	−5.69	0.000*
G2–G4	−2.85	−9.05	3.35	0.689	−4.06	−7.22	−0.89	0.006*
G2–G5	0.05	−6.15	6.25	1.000	−1.43	−4.60	1.72	0.698
G3–G4	0.68	−5.52	6.88	0.998	4.79	1.63	7.96	0.001*
G3–G5	3.58	−2.62	9.78	0.480	7.41	4.25	10.58	0.000*
G4–G5	2.90	−3.30	9.10	0.675	2.62	−0.54	5.78	0.147

*Statistically significant. CI: Confidence interval, HSD: Honestly significant difference, SMH: Surface microhardness

Table 2: Comparison of wt% of Calcium and phosphorous in various groups

Comparison groups	Mean difference wt% Ca	*P* (Tukey HSD)	Mean difference in wt% *P*	*P* (Tukey HSD)	Mean difference Ca/P ratio	95% CI		*P* (Tukey HSD)
						Lower	Upper	
G1–G2	−9.66	0.000*	−4.89	0.000*	−0.09	−0.21	0.03	0.230
G1–G3	−13.38	0.000*	−6.29	0.000*	−0.16	−0.28	−0.03	0.004*
G1–G4	−11.76	0.000*	−5.82	0.000*	−0.11	−0.23	0.01	0.087
G1–G5	−11.46	0.000*	−5.57	0.000*	−0.13	−0.25	−0.01	0.021*
G2–G3	−3.72	0.002*	−1.40	0.253	−0.07	−0.19	0.05	0.486
G2–G4	−2.10	0.176	−0.92	0.653	−0.02	−0.14	0.10	0.989
G2–G5	−1.79	0.315	−0.67	0.854	−0.04	−0.16	0.07	0.823
G3–G4	1.61	0.423	0.47	0.955	0.04	−0.07	0.17	0.779
G3–G5	1.92	0.253	0.72	0.823	0.02	−0.09	0.14	0.979
G4–G5	0.30	0.997	0.24	0.996	−0.02	−0.14	0.09	0.977

*Statistically significant. CI: Confidence interval, Ca/P: Calcium/phosphorous

against MMP-2 and MMP-9.[15] CE (vaccinium macrocarpan) has shown benefits in managing periodontal disease by its inhibitory action on the production and catalytic activity of MMP-1 and MMP-9 in inflamed periodontal tissues.[16]

PAs may have a positive influence on the remineralization process by causing cross-linking of the collagen matrix as the network is stabilized in an expanded state leaving the intrafibrillar spaces open for remineralization.[17] In addition, PA increases the acidic pH of CPP-ACPF that further enhances remineralization.[18]

These properties of PAs can explain the results of the present study where it was found that the groups pretreated

with plant-based dentin biomodifying agents containing PA, being consequently treated with CPP-ACPF showed a synergistic remineralizing effect evidenced by higher SMH values.

The mean SMH values were higher in G3 followed by G4 and G5 [Figure 1a] and the mean difference in % change in SMH was statistically significant when G3 was compared with G4 and G5 ($P = 0.001$ and $P = 0.000$), respectively [Figure 1b and Table 1]. The catechins in PAs are linked with different interflavonoid bonds that categorize them as either A-type or the B-type analogs, the latter showing a relatively higher molecular mass of two units than the former.[19] With increasing molecular weight of PAs, their polarity increases because of the presence of additional hydroxyl groups and therefore possess a higher potential for hydrogen bonding and remineralization.[4] In GSE, the catechins in PAs are mainly linked with C4 → C6 or C4 → C8 interflavonoid bonds, which features it as B-type PAs,[20] whereas the unusual structure of PAs in CE shows A-type linkages, demonstrating a secondary ether linkage between C-2 ring of the upper unit and the A-ring of the lower unit.[21] GTE contains both A-Type and B-Type of PAs in their composition,[22] and this may be responsible for their intermediate performance in improving the SMH as compared to the other two agents in this study.

SEM pictomicrographs of G1 showed enlarged, exposed, and unobstructed dentinal tubules with no obvious sediments [Figure 2a]. The intertubular dentin was partially demineralized, with an exposed peritubular dentin. Most organic matter is concentrated in the intertubular dentin, in contrast, peritubular dentin being hypermineralized with hydroxyapatite crystals dispersed in an organic matrix has high resistance to acidic damage.[23]

SEM pictomicrographs of G2 revealed a thin homogenous layer of amorphous crystal-like deposits coating the dentin surface and occluding the dentinal tubules, thereby reducing their diameter [Figure 2b]. G3, G4, and G5 showed higher incidences of dentinal tubule occlusion by ACP deposits [Figure 2c-e] which were further confirmed as hydroxyapatite crystals in previous studies.[24]

EDAX analysis revealed that Ca/P ratio was highest in G3 (1.89), followed by G5 (1.86), G4 (1.84), G2 (1.82), and G1 (1.73) [Figure 1c]. This difference was statistically significant when G1 group was compared to G3 and G5 ($P = 0.004$ and $P = 0.021$, respectively) [Figure 1d and Table 2]. A Ca/P ratio of 1.6 can facilitate an optimum rate of remineralization with the Ca/P ratio of sound/healthy dentin varying from 1.7 to 2.14.[25] Since the observed ratio in dentin samples treated with the test agents was greater than these values, this implied that the formation of a hypermineralized zone is possible with these agents. Trace amounts of F (Fluorine) were also detected (0.65 wt%) in the all the samples treated with the test agents presumably attributed to the fluoride component of CPP-ACPF (0.2% or equivalent to 900 ppm).

The increased incidence of deposition of hydroxyapatite crystals in G3, G4, and G5 observed through SEM-EDAX analysis corroborate the results of SMH test obtained in this study that showed higher SMH values in these groups. Thus, the null hypothesis is rejected as these findings are suggestive of the positive synergism shown by CPP-ACPF when used after pretreatment with plant-based dentin biomodifying agents, leading to an enhanced potential for remineralization.

In this study, we have been able to establish the greater effectiveness of a dual-step strategy for biomimetic remineralization in managing erosive demineralization of dentin using an *in vitro* model. Clinically, this not only aligns with the concept of minimal intervention dentistry but also promises potential benefits such as alleviation of dentinal hypersensitivity and superior resistance to degradation of collagen matrix, thus providing a more reliable substrate for adhesive restorations. The topical application of plant-based extracts as bio-modifying agents in the form of mouthwashes, topical gels, and formulations has already been clinically evaluated in a similar context. The dual-step treatment strategy in this study used appropriate concentrations and application times for the various test agents. However, the clinical success of this strategy requires further refinement of standardization in terms of the choice of optimum concentrations, modes, and times of application of these test agents as options for future research.

Limitations of the study

In this study, the analysis was done immediately posttreatment, and the consideration of long-term stability of the collagen matrix and the deposited mineral layers were beyond the purview of this study. Further time-bound *in vivo* studies may be considered to assess if the hardened, remineralized hydroxyapatite and fluorapatite layers formed can withstand the challenges produced in clinical and *in vivo* conditions and remain stable for long periods without undergoing degradation.

CONCLUSIONS

Within the limitations of this study, it can be inferred that a synergistic effect of CPP-ACPF may exist when used after pre-treatment with plant-based dentin biomodifying agents containing PA on the remineralization of eroded dentin.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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