



## COMPARATIVE EVALUATION OF THE EFFECT OF VARIOUS REMINERALIZING AGENTS ON ROOT DENTIN: AN IN VITRO STUDY

### Dental Science

<b>Dr Jyothi Shetty</b>	Post Graduate student Department of Conservative Dentistry and Endodontics A B Shetty Memorial Institute of Dental Sciences Mangalore, Karnataka, India
<b>(Dr) Darshana Devadiga*</b>	Professor Department of Conservative Dentistry and Endodontics A B Shetty Memorial Institute of Dental Sciences Mangalore, Karnataka, India. * Corresponding Author
<b>Dr. Mithra N. Hegde</b>	Vice Principal and Head, Department of Conservative Dentistry and Endodontics A B Shetty Memorial Institute of Dental Sciences, Mangalore, Karnataka, India

### ABSTRACT

50 root dentin sections were obtained from 25 extracted premolars and divided randomly into 5 groups of 10 samples each. Except group 1, all the other group specimens were demineralised by immersing in a demineralising solution for 96 hours at 37°C. The specimens were then subjected to surface treatment with grape seed extract, silver diamine fluoride and calcium sucrose orthophosphate complex according to the groups. The samples were stored in artificial saliva during the intervals and subjected to pH cycling and evaluated using SEM-EDAX and the results statistically compared.

The tested remineralizing agents showed statistically significant increase in remineralization when compared to the demineralized group. Calcium sucrose orthophosphate showed the highest Ca/P values followed by Silver diamine fluoride and grape seed extract.

### KEYWORDS

Grape seed extract (GSE), Silver diamine fluoride, calcium sucrose orthophosphate complex (CSOPC), SEM EDAX.

### INTRODUCTION

With the improvement in the field of oral health care, the rate of edentulism has reduced significantly over the years. However with the increased number of natural teeth present in the elderly population, the risk and prevalence of caries have increased.<sup>1</sup> There is also a concomitant increase in the prevalence of periodontal disease, gingival recession resulting from periodontal diseases leave the root surfaces exposed to the development of root caries, which has important implications for preventive and restorative treatments.<sup>2</sup>

The concept of "prevention of extension" by remineralization of caries is highly desirable and is one of the cornerstones of minimal invasive dentistry.<sup>3</sup> Caries starts as demineralization followed by destruction of organic matter and invasion by microorganisms.<sup>4</sup> Remineralization is the repair of incipient carious lesions. Structurally intact collagen provides a good scaffold to promote remineralization. An agent which strengthens collagen – based tissues as well as increases collagen cross-links would promote remineralization.<sup>5</sup>

In Minimum intervention dentistry strong emphasis is placed on arresting and reversing incipient lesions by using suitable remineralizing agents.<sup>6</sup> The dentin is highly susceptible to degradation by proteolytic enzymes due to the presence of high collagen matrix and decreased mineral content.<sup>7</sup> Thus, novel strategies should be developed to promote remineralization by preserving the organic matrix which aids in the reduction of the progression of root caries.<sup>8</sup>

Although fluoride has a remarkable potential of remineralizing enamel, its effect on dentin seems to be relatively less effective.<sup>9</sup> Natural products have been used as folk medicines for many years and are promising sources for novel therapeutic agents. A majority of the natural products have been studied in the field of oral health focusing on their antibacterial activities but very few have been reported on the process of demineralization and remineralization of oral hard tissues.<sup>10</sup>

Polyphenols are plant-derived substances which have antioxidant and anti-inflammatory properties. One such polyphenol is Proanthocyanidin (PA) which is a bioflavonoid-containing molecular nucleus made of benzene-pyran-phenolic acid.<sup>11</sup> Common sources of polyphenols are fruits, vegetables, flowers, seeds, and tree barks. The PA present in grape seed extract (GSE) accelerates the process of conversion of soluble collagen to insoluble collagen and increases collagen synthesis, thus forms a scaffold for remineralisation.<sup>11</sup>

Calcium sucrose phosphate – calcium orthophosphate complex (Toothmin™) is a remineralizing agent that is based on "Anticay" technology. It supplies both calcium and phosphate in a soluble form and by a common ion effect reduces the acid solubility of enamel thereby increasing the rate of remineralization.<sup>12</sup> In addition, sucrose phosphate ions adsorb onto the enamel surface and cause decrease in the rate of acid dissolution and also acts as a complement to fluoride. Its effective remineralizing action is because of its solubility in water providing high concentrations of free calcium and phosphate several times higher than normally present in saliva.<sup>12</sup>

Silver diamine fluoride (SDF 38%) contains high concentration of silver and fluoride ions where silver acts as an antimicrobial agent and fluoride promotes remineralization. It has the dual action of limiting the caries process as well as prevention of new caries formation. It has been reported that when SDF reacts with the hydroxyapatite of the tooth, it releases calcium fluoride and silver phosphate, these reaction products help in the prevention and arresting of dental caries.<sup>13</sup>

Modern prospective caries studies emphasis on the estimation of small changes in the mineral content of the tooth. Scanning electron microscopy with an energy dispersive x-ray analysis attachment is one such microanalytical technique.<sup>14</sup> It is a combined methodology used to visually study the surface morphology as well as qualitatively assess the mineral content present in the sample.<sup>14</sup>

In the past, several studies have been conducted on evaluation of the remineralizing potential of agents such as fluoride and CPP –ACP products mainly on the enamel but very few on root dentin. Hence, the present study aimed at studying the remineralization potential of alternative agents like grape seed extract, calcium sucrose orthophosphate complex (Toothmin™) and silver diamine fluoride 38% (Saforide™) on root dentin.

### Aim and objective of the study:

-To evaluate the remineralization potential of remineralizing agents such as grape seed extract, Silver diamine fluoride (38% Saforide™) and calcium sucrose orthophosphate complex (ToothMin™) on root dentin using Scanning Electron Microscopy (SEM) –Energy Dispersive X-ray Analysis [SEM- EDAX].

### Materials and Method:

The study was conducted in the Department of Conservative Dentistry and Endodontics, A.B Shetty Memorial Institute of Dental Sciences, Mangalore and evaluation was done at DST PURSE LAB, Mangalore University, Mangalore.

**Source of Data:**

Freshly extracted intact premolars from patients in the age group 14 - 18 years, extracted for orthodontic purposes were collected. As per the guidelines and recommendations by Occupational Safety & Health Administration (OSHA), the teeth were disinfected and stored.

**Specimen Preparation**

**Root dentin sample preparation:**

50 root dentin sections were obtained below the CEJ measuring about 4x4x3mm using a low speed diamond cutting disc with adequate water coolant. An impermeable surface coating material like nail varnish was applied on all the surfaces except the front surface.

**Procedure**

All the specimens were randomly divided into 5 groups of 10 specimens each.

Except group 1, all the specimens were immersed in demineralising solution for 96 hours at 37°C. Specimens were stored in artificial saliva and subjected to surface treatment as follows:

**Group 1** - (Control group) – no surface treatment, stored in artificial saliva only.

**Group 2** - only demineralisation, no surface treatment with remineralising agent.

**Group 3** – Demineralisation and surface treatment with Grape seed extract (prepared in phosphate buffer at pH 7.4)

**Group 4** – Demineralisation and surface treatment with Silver diamine fluoride (38%, Saforide™).

**Group 5** – Demineralisation and surface treatment with Calcium sucrose orthophosphate complex (ToothMin™, Abboth Pharmaceuticals).

**Protocol for Root Dentin Demineralization:**

The samples of all the Groups except Group I (control) were individually immersed in the demineralizing solution for a period of 96 hours at 37°C.

**Protocol for Root Dentin Remineralization:**

After demineralization, the samples of Group 3, Group 4 and Group 5 were subjected to surface treatment with their respective remineralizing agents at 37°C for a period of 14 days. During the intervals, the samples were kept in artificial saliva.

**pH cycling:**

To simulate the oral conditions, the samples were subjected to a pH cycling regime. In this study, pH cycling protocols described by Featherstone et al in

1986 was employed which simulates in vivo high caries risk condition. The stimulation of active cycles of demineralization and remineralization was done by sequential immersion of the root specimens in acidic (pH 4.3) and remineralizing buffer solutions. The remineralization solution is composed of calcium and phosphate at a known degree of saturation which approximates the qualities of saliva.

**Evaluation of the specimens:**

All the prepared specimens were subjected to evaluation using Scanning electron microscopy – Energy Dispersive X-ray analysis [SEM –EDAX] at DST PURSE Lab, Mangalore University, Mangalore.

Data obtained was compared and statistically analysed using One-way Anova and Tukey's Post-hoc test.

**RESULTS**

**Table 1 – Mean and Standard deviation of Calcium and Phosphate values in all groups ( by EDAX evaluation).**

Groups		N	Mean	Std. Deviation	Mean Difference	t	p	95% Confidence Interval of the Difference	
								Lower	Upper
Control (Grp 1)	Ca	10	25.26	1.24	5.80	12.047	<0.001	4.79	6.81
	P	10	19.45	0.89					
Demineralised (Grp 2)	Ca	10	18.25	0.93	4.15	11.417	<0.001	3.39	4.91
	P	10	14.10	0.68					
GSE (Grp 3)	Ca	10	21.81	0.66	6.75	25.170	<0.001	6.19	7.32
	P	10	15.05	0.53					
SDF (Grp 4)	Ca	10	23.20	0.71	7.67	29.451	<0.001	7.12	8.22
	P	10	15.53	0.42					
CSOPC (Grp 5)	Ca	10	24.26	1.30	7.91	14.069	<0.001	6.73	9.09
	P	10	16.35	1.22					

**Table 2 – Multiple Intergroup Comparisons for Calcium using Tukey HSD test**

Dependent Variable: Calcium					
(I) group	(J) group	Mean Difference (I-J)	p	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	Demineralised	7.00	<0.001 ***HS	5.73	8.28
	GSE	3.45	<0.001 ***HS	2.17	4.72
	SDF	2.06	<0.001 ***HS	0.78	3.33
	CSOPC	0.99	0.192 NS	-0.28	2.27
Demineralised	GSE	-3.56	<0.001 ***HS	-4.83	-2.29
	SDF	-4.95	<0.001 ***HS	-6.22	-3.68
	CSOPC	-6.01	<0.001 ***HS	-7.28	-4.74

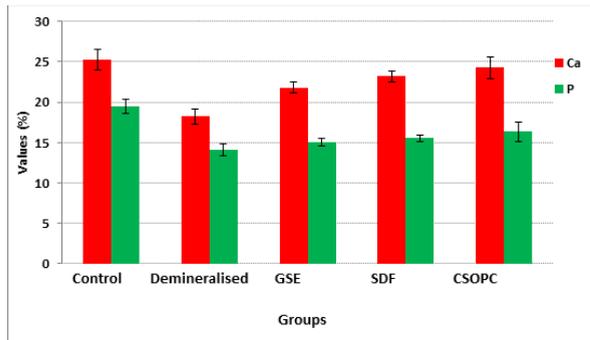
GSE	SDF	-1.39	0.026 *S	-2.66	-0.12
	CSOPC	-2.45	<0.001 ***HS	-3.73	-1.18
SDF	CSOPC	-1.06	0.142 NS	-2.33	0.21
NS- Not significant. ***HS – Highly significant p<0.001. *S – significant p<0.05					

**Table 3- Multiple Intergroup Comparisons for Phosphate using Tukey HSD test**

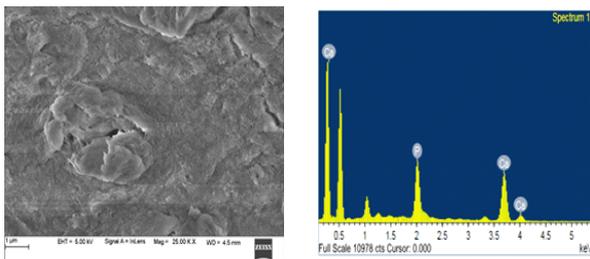
Dependent Variable: Phosphate					
(I) group	(J) group	Mean Difference (I-J)	p	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	Demineralised	5.35	<0.001 ***HS	4.34	6.37
	GSE	4.40	<0.001 ***HS	3.38	5.41
	SDF	3.92	<0.001 ***HS	2.91	4.94

	CSOPC	3.10	<0.001 ***HS	2.09	4.12
Demineralised	GSE	-0.95	0.075 NS	-1.97	0.06
	SDF	-1.43	0.002 *S	-2.45	-0.42
	CSOPC	-2.25	<0.001 ***HS	-3.26	-1.23
GSE	SDF	-0.48	0.669 NS	-1.49	0.54
	CSOPC	-1.29	0.006 *S	-2.31	-0.28
SDF	CSOPC	-0.82	0.168 NS	-1.83	0.20

NS- Not significant. \*\*\*HS –Highly significant p<0.001. \*S –significant p<0.05



Graph 1: Mean and Standard deviation of calcium and Phosphate values in five different groups (EDAX)



Mean values of calcium is highest in Control group (25.26+ 1.24) followed by CSOPC (24.26+\_1.30), SDF(23.20+\_0.71) and GSE (21.81+\_0.66).

2. Mean Values of calcium is least in Demineralised group (18.25+ 0.93). There is overall statistical significance in calcium values between the 5 groups (P<0.001).

3. Mean values of Phosphate is highest in Control group(19.45+ 0.89), followed by CSOPC (16.35+\_1.22), SDF(15.53+ \_0.42)and GSE(15.05+\_0.53).

4. Mean values of Phosphate is least in demineralised group(14.10+\_0.68). There is overall statistical significance in phosphate values between the 5 groups (P<0.001).

Fig 9: Group 1 (Control) -a) SEM image of intact root dentin. b) Elemental analysis of intact root dentin by EDAX

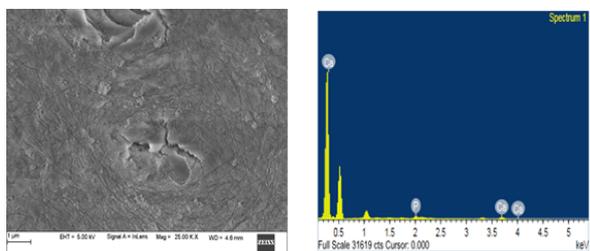


Fig 10: Group 2 (Demineralized) –a) SEM image of demineralized root dentin

b) Elemental analysis of demineralized root dentin by EDAX

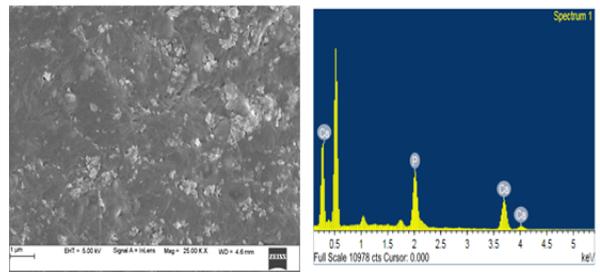


Fig 11: Group 3: a) SEM image of root dentin surface treated with GSE shows deposits on the surface. b) Elemental analysis of root dentin treated with GSE by EDAX.

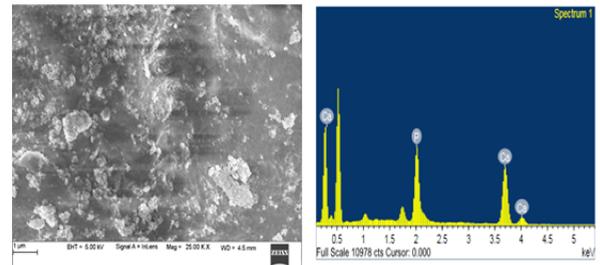


Fig 12: Group 4 a) SEM image of root dentin surface treated with Silver diamine fluoride Showing thick deposits on surface. b) Elemental analysis of root dentin treated with Silver diamine fluoride by EDAX.

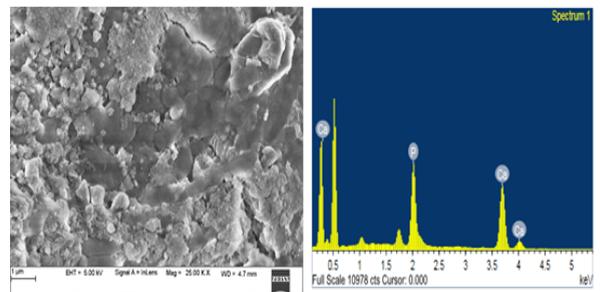


Fig 13: Group 5 –a) SEM image of root dentin surface treated with calcium sucrose orthophosphate complex showing thick deposits on the surface. b) Elemental analysis of root dentin surface treated with CSOPC by EDAX.

**Discussion**

In the present study, the effect of a natural remineralizing agent like grape seed extract was compared with synthetic agents like silver diamine fluoride and calcium sucrose orthophosphate complex on demineralized root dentin quantitatively using Energy Dispersive X-ray Analysis [EDAX] to estimate the Ca/P values and qualitatively using SEM to study the surface morphology. Amongst the test groups, the Calcium sucrose ortho phosphate group (Gp 5) showed the highest Ca/P content followed by Silver diamine fluoride (Gp 4) and Grape seed extract (Gp 3) respectively. Amongst the control groups, the intact root dentin (Gp 1) showed the highest Ca/P values while demineralized group (Gp 2) without any surface treatment with remineralizing agent showed the lowest values.

When compared to the demineralized control group without surface treatment (Gp 2) all the test groups surface treated with different remineralizing agents showed a definite improvement in Ca/P values; but the recovery was not equivalent to that of Ca/P values of intact root dentin of the control group (Gp1). The improvement was statistically significant when compared to Gp 2 (demineralised) and is in agreement with several other studies.<sup>11,12,16,23,28,34.</sup>

Dentin is a complex, hydrated vital tissue that is shielded by overlying enamel and periodontal tissues, however it can get exposed following the loss of enamel or due to recession of gingiva.<sup>54,55</sup> Once the dentin is exposed, it is susceptible to demineralization and remineralization as it is placed in equilibrium with the surrounding aqueous environment.<sup>56</sup> The process of dentin remineralisation is

complicated as it involves the interaction of minerals with collagen and non-collagenous proteins.<sup>57</sup> In demineralised dentin, the collagen based organic matrix is exposed. 90% of the organic matrix is made of fibrillar type I collagen whereas the remaining 10% is made of non-collagenous proteins. Therefore, the remineralisation of dentin depends on the interactions of collagen matrix with the mineral crystallites.<sup>36,37</sup>

Three conditions are necessary to remineralise the demineralised dentin.<sup>37</sup> First, residual mineral crystals should be present to serve as growth centres. Second, there should be a supply of calcium and phosphorous enriched mineral sources and finally, there should be sound collagen structure to act as scaffold for the deposition and growth of mineral crystals. Therefore for the remineralization of demineralised dentin an environment that favours calcium and phosphorous precipitation is essential.<sup>37</sup> Hence, in treating dentinal hypersensitivity, cervical dentin wear and root caries it is important to enhance the remineralisation of dentin.<sup>52</sup>

Among the control groups, Gp 2(demineralised) showed the lowest Ca/P values when compared to Gp1(intact) and is statistically significant. The demineralization of root dentin was carried out with a demineralizing agent of pH 5.0 as used by Featherstone (1983)<sup>38</sup> in a study which showed that acids diffuse into enamel, dentin and cementum, partially dissolving the mineral crystals (composed of carbonated hydroxyapatite) to release calcium and phosphate ions to produce artificial caries- like lesion.<sup>39</sup>

The samples treated with CSOPC showed the highest Ca/P values which was statistically significant compared to the Control Group Gp 2 (demineralised) but not when compared with Gp 1(Intact) and other experimental groups treated with GSE and SDF respectively. As there are no previous studies published on the effect of CSOPC on root dentin, the results of this group cannot be compared to other studies. However, similar study on Enamel reported by Kaur et al<sup>32</sup> attributed the remineralising ability of CSOPC to the Anticay technology on which Toothmin<sup>TM</sup> system is designed. Anticay is a mixture of calcium sucrose phosphate and inorganic calcium phosphate consisting of 10-12% calcium and 8-10% phosphorous by weight.<sup>12</sup> Calcium sucrose phosphate decreases tooth demineralization and promotes remineralization. Its effective remineralizing action is because of its solubility in water providing high concentration of free calcium and phosphate ions several times higher than normally present in saliva. This complex helps reduce the acid solubility of dentin and increases remineralization rate by a common ion effect.<sup>12</sup>

The samples treated with SDF showed the second highest Ca/ P values which was statistically significant compared to the GP 2 (demineralised) and Gp 1 (intact); but not statistically significant compared with Gp 3(GSE) and Gp 5(CSOPC). This is in agreement with study results by Mei et al<sup>34</sup> who concluded that application of 38% SDF had an inhibitory effect on demineralization and preserved collagen from degradation in demineralized dentin. Saforide<sup>TM</sup> (38% silver diamine fluoride/ SDF [Ag (NH<sub>3</sub>)<sub>2</sub>F]) contains 44,800 ppm fluoride and 2,53,900 ppm silver. It has been reported that when SDF reacts with the tooth hydroxyapatite, it releases calcium fluoride and silver phosphate, these reaction products aids in remineralisation. The silver phosphate forms insoluble precipitates on the tooth surface. The calcium fluoride formed supplies a reservoir of fluoride for the formation of flourapatite which is more acid attack resistant than the hydroxyapatite.<sup>51</sup>

The samples treated with GSE showed lowest Ca/P values which was statistically significant compared to the control groups Gp 2 (demineralised) and Gp 1 (intact); but not statistically significant on comparing with Gp 4 (SDF) and Gp 5 (CSOPC). This was in accordance to studies conducted by Episighne et al<sup>27</sup> and Pavan et al<sup>48</sup> who stated that grape seed extract inhibits demineralization of artificial carious lesions in both enamel and dentin, but in a different way in each structure and in a smaller scale when compared to fluoride. Proposed mechanism of its action is that it contributes to the deposition of mineral on the superficial layer of the lesion. When mixed with remineralizing solutions at pH 7.4, GSE is said to form visually insoluble complexes. GSE stabilises the exposed collagen matrix by interacting with the organic component of root dentin through PA-collagen interaction.

It has been demonstrated that the high PA content in GSE increases the

synthesis of collagen as well as accelerates the conversion process of collagen from soluble to insoluble state and positively effects the remineralization of artificial root caries.<sup>11</sup>

The use of 6.5% PA effectively contributes to the remineralization process of artificial root caries, by incorporating calcium ions from the remineralization solution into the caries lesion. The cross-linked collagen matrix can act as a scaffold for the deposition of minerals. Additionally, collagen cross- linked by PA enhances the resistance to degradation by enzymes as well as increases the collagen stability. Apart from this, the ion chelation mechanism of PA helps in binding and aggregation of calcium ions thereby enhancing remineralization.

Studies conducted by Episinghe et al on the combined use of PA and CPP-ACFP had a synergistic effect of enhancing mineral gain as well as increasing the hardness of artificially induced root caries, thereby promoting remineralization.<sup>18</sup> Hence, use of GSE with a mineral based remineralising agent would be more effective as it would stabilise the organic component of the root dentin as well as increase the mineral deposition. In the present study only GSE was used, this might be one of the possible reason for decreased Ca/P values compared to the other two remineralizing agents in this study.

To stimulate the intra-oral conditions, artificial saliva was used as an storage medium during experimental procedure and the samples were subjected to pH cycling. However, using artificial saliva alone cannot model remineralisation and demineralisation process in the intra oral environment which is also related to various other biological factors such as biofilm and pellicle which offers greater protection to the tooth surfaces thereby resisting demineralization. Therefore, the specimens in this study were subjected to a procedure of pH cycling to further simulate the intra- oral conditions.

To study various changes such as detection of chemical changes taking place in enamel and dentin during demineralization and remineralization, it is necessary to carry out atleast part of the experiment under in-vitro conditions using direct and indirect techniques available like micro-radiography, micro-tomography, atomic absorption photospectrometry, scanning electron microscope, scanning electron microscope energy dispersive x-ray analysis and surface microhardness. In the present study, Energy Dispersive X-Ray analysis [EDAX], a microanalytic technique for analysis of elements at the ultra structural level was used for analysis of calcium and phosphorous content of the root dentin samples in combination with scanning electron microscopy [SEM] for surface micro-structural analysis as described in several studies.<sup>44</sup>

In SEM analysis, the sample surface is scanned with a focused beam of electrons which interact with atoms on the surface, producing various signals that can be detected and contain information about its topography and composition to produce an image with a resolution better than 1 nanometer. The most common mode of detection is by secondary electrons emitted by atoms excited by the electron beam. SEM requires proper specimen preparation and examination conditions. It has been widely used in several studies to observe the structural surface changes on tooth during erosion and demineralization.<sup>45,46,47</sup>

The advantage of an in-vitro model is that it provides well-controlled experimental conditions to induce demineralization-remineralization; however the results of this in-vitro study may not fully represent the in-vivo conditions; wherein the oral cavity is constantly flushed with saliva containing lipids, carbohydrates, proteins and minerals. Hence, further studies that closely stimulate in-vivo conditions followed by long term clinical trials should be undertaken so as to corroborate the above mentioned results as well as to determine the long term effects of these materials in the oral environment.

### Conclusion

Within the limitation of this in vitro study, it can be concluded that all the tested remineralizing agents showed effective remineralizing of the demineralized root dentin in the following order: calcium sucrose orthophosphate complex, silver diamine fluoride and grape seed extract. Amongst the remineralizing agents tested in the study, calcium sucrose orthophosphate complex was found to be marginally more effective than silver diamine fluoride and grape seed extract but was not statistically significant.

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