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Comparative evaluation of remineralization potential of a formulated non-fluoridated grape seed proanthocyanidin rich dentifrice with fluoride and CPP-ACP dentifrice on primary teeth an *in-vitro* study

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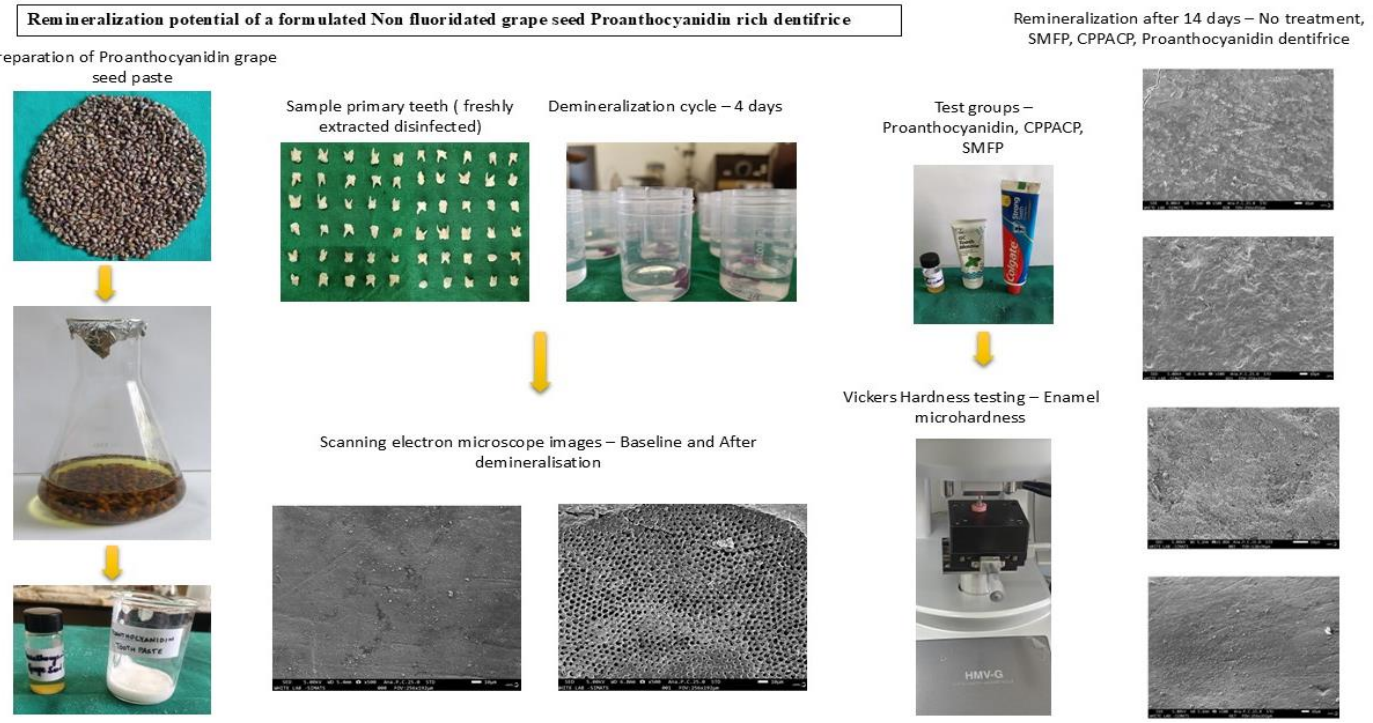
ABSTRACT

The objective of this study was to evaluate and compare the remineralization potential of a formulated non fluoridated gape seed Proanthocyanidin rich dentifrice with commercially available fluoridated and non-fluoridated dentifrices on primary teeth in an invitro environment. Sixty freshly extracted primary molars were demineralized using glacial acetic acid for four days and then divided into four groups (n=15):



Group I: No remineralization treatment (control); Group II: Sodium monofluorophosphate dentifrice; Group III: Casein phosphopeptide-amorphous calcium phosphate(CPP-ACP); Group IV: Proanthocyanidins dentifrice from grape seed extract. All treatments were applied for 3 minutes, twice daily, over 14 days. Teeth were immersed in artificial saliva during the remineralization period. Vickers enamel microhardness (VEMH) testing and scanning electron microscopy (SEM) were performed at baseline, post-demineralization, and post-remineralization. Statistical analysis was conducted using one-way ANOVA and Tukey's HSD test. VEMH values indicated significant differences among the groups after remineralization (p=0.00). Group IV demonstrated the highest mean VEMH values, indicating superior remineralization, while Group I showed the lowest values. Post Hoc analysis revealed significant differences between Group IV and all other groups. SEM images of Group IV displayed a dense, homogenous, globular surface resembling sound enamel, with no porosities, confirming effective remineralization of the demineralized enamel matrix. Proanthocyanidins dentifrice from grape seed extract demonstrate remarkable remineralization potential, comparable to fluoridated agents. These findings suggest Proanthocyanidins dentifrice as a viable non-fluoridated alternative for enamel remineralization in primary teeth.

Keywords: Proanthocyanidins, remineralization, Primary teeth, Enamel micro hardness, Grape seed extract, Non-fluoridated agents.

INTRODUCTION

Dental caries, commonly known as tooth decay, is a pervasive chronic disease affecting individuals across all age groups. It results from the interaction of multiple factors, including dietary sugars, bacterial activity, host susceptibility, and time, leading to the breakdown of the mineralized structure of teeth. Caries is not merely a visible cavity; it begins as an invisible process of demineralization beneath the enamel surface. The initial clinical manifestation of demineralization is often seen as a white, chalky spot, which represents reversible enamel damage if addressed promptly [1, 2].

Remineralization, the natural repair process for early caries lesions, occurs when lost minerals are replenished, typically in a neutral pH environment. Saliva plays a crucial role in this process by providing calcium, phosphate, and fluoride to restore the hydroxyapatite matrix of enamel. However, in an acidic oral environment, the pH drops, impairing remineralization and leading to further mineral loss. Fluoride has long been recognized for its ability to inhibit demineralization and promote remineralization by forming acid-resistant Fluor apatite crystals on enamel surfaces [3].

India bears the highest global prevalence of dental caries in both permanent and primary teeth [4]. Fluoride-based interventions, including fluoridated water, dentifrices, and professional applications, have significantly reduced caries incidence in developed nations. Sodium monofluorophosphate (SmFP), commonly used in fluoridated dentifrices, is particularly effective in caries prevention [5, 6]. However, India's groundwater often contains fluoride levels exceeding the World Health Organization's recommended threshold of 1.5 mg/L, averaging 2.37 mg/L, posing a risk of dental and skeletal fluorosis [7]. Additionally, accidental ingestion of fluoride by children can lead to acute toxicity and dental fluorosis, raising concerns about its safety [8].

The search for safer alternatives has led to the exploration of non-fluoridated remineralization agents. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), derived from milk protein, has shown notable remineralization potential by creating a calcium- and phosphate-rich environment around enamel surfaces [9].

However, its milk-derived nature poses risks for children with allergies, including severe reactions such as anaphylaxis [10].

Proanthocyanidins (PACs), naturally occurring polyphenolic compounds found in fruits, seeds, and bark, particularly grape seeds, offer a promising alternative. PACs facilitate remineralization by forming mineral deposits and cross-linking on carious lesions, thereby preventing calcium and phosphate loss under acidic conditions [11, 12]. Grpe seed extract, rich in PACs, has shown potential for promoting enamel repair with minimal adverse effects.

This study aimed to evaluate and compare the remineralization potential of a formulated non-fluoridated grape seed proanthocyanidin-rich dentifrice with fluoridated dentifrice (SmFP) and non-fluoridated CPP-ACP dentifrice on artificially induced carious lesions in primary teeth using in vitro methods.

MATERIALS AND METHODS

Formulation of the Grape Seed Proanthocyanidin Dentifrice

Proanthocyanidins were extracted using the maceration method from shade-dried grape seeds, where the seeds were soaked in 10% ethyl acetate, followed by purification and filtration to obtain the unrefined extract (Figure 1). The compounds were then identified and confirmed using gas chromatography coupled with mass spectrometry (GC-MS). An SLS-free, proanthocyanidin-rich dentifrice was formulated using calcium diphosphate, sodium cocoyl glutamate (SLS-free surfactant), water, silica gel, carboxymethyl cellulose, propylene glycol, sorbitol, sodium saccharin, and 0.1mL of proanthocyanidin liquid extract (Figure 2). This formulation incorporates proanthocyanidin as the primary active ingredient, avoiding the use of potentially irritating surfactants like sodium lauryl sulfate.

Sample Size

A priori power analysis for a one-way ANOVA with fixed effects was conducted using G*Power software 3.1, yielding a required sample size of 54 based on an effect size of f = 0.50 and a power of 0.90. The sample size was rounded to 60, with 20 samples per group,

and ethical clearance was obtained from the Institutional Human Ethics Committee [CARE-IHEC-I/1243/22].

Figure 1: a – Grape seeds soaked in ethyl acetate solution. b – Crude proanthocyanidin extract

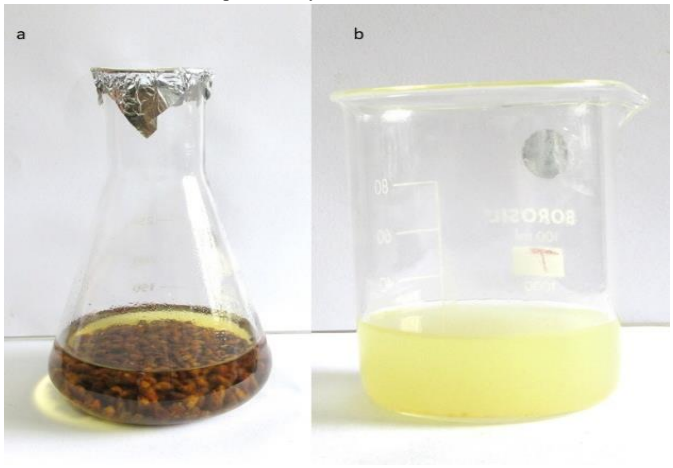
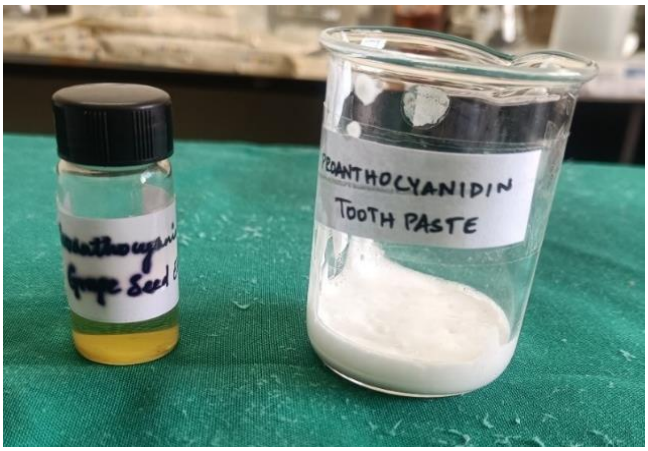


Figure 2: Proanthocyanidin extract and formulated dentifrice



Preparation of tooth samples

Sixty primary molars with intact buccal and lingual/palatal surfaces, as well as caries-free molars scheduled for extraction, were included in the study after obtaining parental consent. The extracted teeth were thoroughly cleaned, with soft tissue debris removed using a scaler, and then disinfected by soaking in 10% formalin for two days. A 0.3 x 0.3 cm square window was created on the intact buccal or lingual/palatal surfaces using cellophane tape, while the remaining tooth surfaces were coated with a 25% phthalate solution.

Baseline Micro hardness Testing and Scanning Electron Microscopy [SEM] Data

Vickers enamel micro hardness (VEMH) testing was conducted on all sample surfaces to determine the mean surface hardness of the enamel before creating artificial caries-like lesions. A load of 1.96 N was applied for 15 seconds, three times per surface. Additionally, scanning electron microscopy (SEM) was used to examine the baseline enamel topography of the teeth.

Artificial Caries Formation

Demineralization

To induce artificial caries-like lesions, the prepared tooth samples were subjected to an acidic challenge by immersing them in a demineralizing solution for four days. The demineralizing solution contained glacial acetic acid, disodium hydrogen phosphate, and calcium chloride dehydrate, with the pH adjusted to 4.5 using potassium hydroxide. After four days of demineralization, the samples were rinsed with deionized water, dried, and stored in sterile containers. Post-demineralization, VEMH testing and SEM analysis were performed again to assess the surface changes.

Experimental Groups and Remineralization Treatment

The samples were divided into three groups: Group I (Control) received no remineralization agent, Group II was treated with fluoride dentifrice (Colgate), Group III treated with CPPACP dentifrice (GC tooth mousse), and Group IV was treated with proanthocyanidin (PAC) dentifrice (Figure 3). After 24 hours of immersion in artificial saliva, the samples were treated twice daily for seven days using the test and control agents, applied for 3 minutes, followed by rinsing with deionized water and re-immersion in fresh artificial saliva. VEMH testing was repeated post-treatment, with three indentation marks made on each sample for 15 seconds at a force of 1.96 N. SEM analysis was conducted to examine the structural changes on the enamel surfaces after remineralization.

Figure 3: Test groups - Proanthocyanidins dentifrice, CPP-APC dentifrice, and Sodium monofluorophosphate dentifrice.

RESULT

Quantitative Analysis of Tooth Enamel Hardness with Vickers Micro hardness Testing

The surface micro hardness of tooth enamel was evaluated at three stages: baseline, post-demineralization, and post-remineralization, using Vickers enamel micro hardness (VEMH) testing. The results, analysed with one-way ANOVA and post hoc Tukey HSD tests, showed that there were no significant differences in the VEMH values between the test and control groups at baseline ($p = 0.332$), nor after demineralization over four days. However, a statistically significant difference was observed between the groups following the remineralization phase ($p = 0.00$) (Table 1).

Table 1: ANOVA test for enamel microhardness – at baseline, after demineralization and after remineralization

		Sum of Squares	df	Mean Square	F	Significance
Baseline	Between Groups	943.117	3	314.372	1.164	.332
	Within Groups	15123.733	56	270.067		
	Total	16066.850	59			
Demineralization	Between Groups	61.383	3	20.461	.269	.847
	Within Groups	4257.600	56	76.029		
	Total	4318.983	59			
Remineralization	Between Groups	356880.933	3	118960.311	592.474	.000
	Within Groups	11244.000	56	200.786		
	Total	**368124.933**	**59**			

Table 2. Enamel microhardness post hoc tukey honestly significant test – remineralization

I	II	-175.533*	5.174	.000	-189.23	-161.83*
	III	-161.000*	5.174	.000	-174.70	-147.30*
	IV	-192.267*	5.174	.000	-205.97	-178.57*
II	I	175.533*	5.174	.000	161.83	189.23*
	III	14.533*	5.174	.034	.83	28.23*
	IV	-16.733*	5.174	.011	-30.43	-3.03*
III	I	161.000	5.174	.000	147.30	174.70
	II	-14.533	5.174	.034	-28.23	-.83
	IV	-31.267	5.174	.000	-44.97	-17.57
IV	I	192.267	5.174	.000	178.57	205.97
	II	16.733	5.174	.011	3.03	30.43
	III	31.267	5.174	.000	17.57	44.97

Among the groups, the highest mean VEMH value was recorded in Group IV, which received the proanthocyanidin dentifrice derived from grape seed extract, indicating superior remineralization efficacy. In contrast, the lowest mean VEMH value was observed in Group I, which received no remineralization treatment. The post hoc Tukey HSD test further confirmed a significant difference between Group IV and the other groups (I, II, and III), highlighting the effectiveness of proanthocyanidin dentifrice in enhancing enamel hardness after remineralization (Table 2).

Visual Evaluation of Enamel Microstructure using Scanning Electron Microscopy [SEM]

Figure 4. SEM images at 500X at different stages

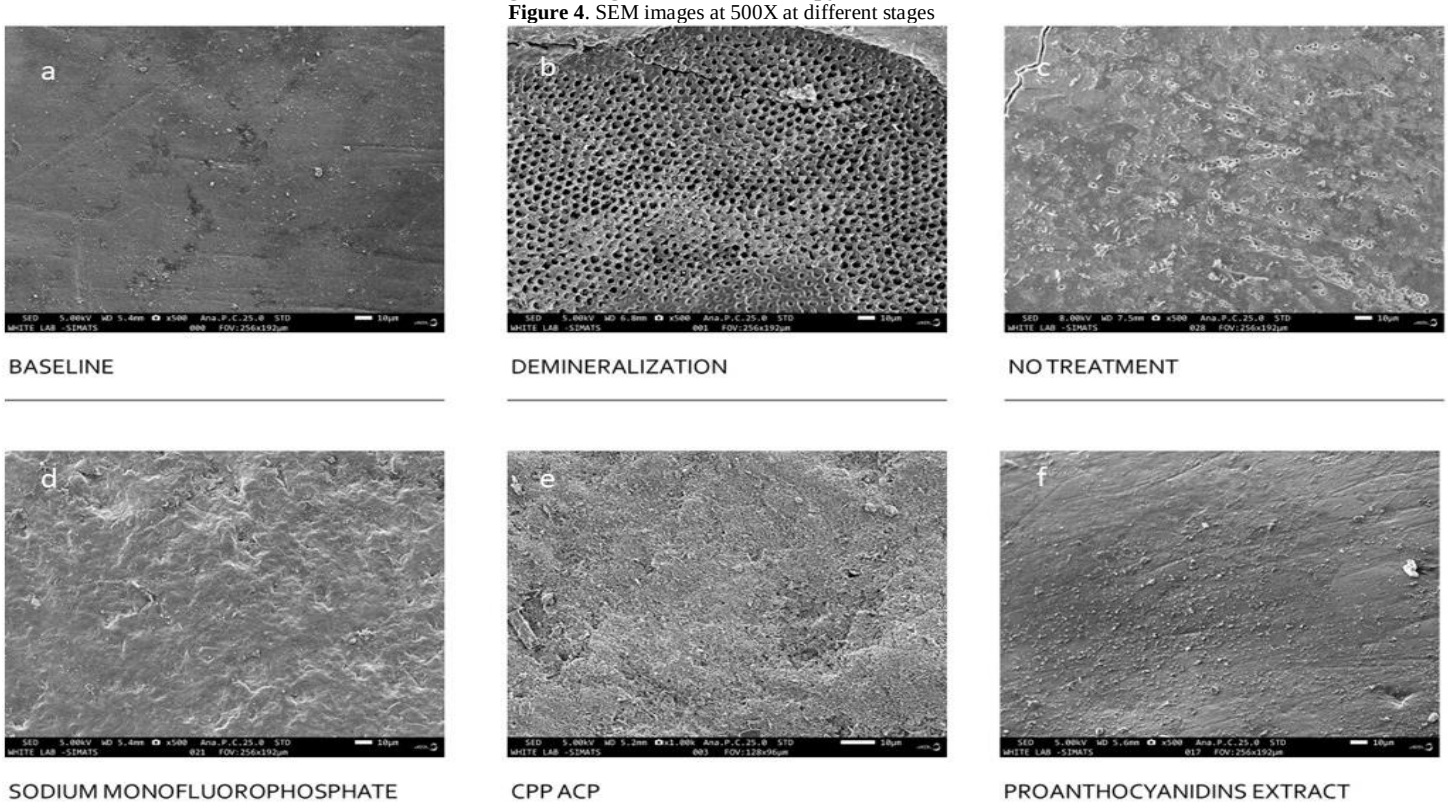


Figure 4 (a-f) illustrates the ultra-structural changes in tooth enamel observed through scanning electron microscopy (SEM) at 1000X magnification at various stages: Before demineralization, after demineralization, and following remineralization with different treatments. Prior to demineralization, the enamel surface (Figure 4a) displayed a smooth texture with spherical particles and minimal irregularities, indicative of healthy enamel. After demineralization for Conversely, enamel treated with CPP-ACP dentifrice (Figure 4e) presented a homogeneous, granular coating with well-organized porosities

Notably, enamel treated with proanthocyanidins dentifrice (Figure 4f) closely resembled sound enamel, featuring a dense, globular appearance with minimal porosities, demonstrating the efficacy of proanthocyanidins in restoring enamel to its near-original state.

DISCUSSION

Dental caries is a progressive disease driven by multiple factors, including plaque, sugar, and acidity, leading to the breakdown of enamel structure. The initial presentation as white, opaque lesions represent subsurface enamel demineralization, which remains reversible through remineralization interventions [13]. Fluoride, widely regarded as the gold standard, promotes remineralization by forming acid-resistant Fluor apatite, inhibiting demineralization, and suppressing bacterial activity by disrupting their enzymatic processes [14].

Fluoride is administered systemically through water, salt, milk fluoridation, or supplements, with water fluoridation being the most effective method for reducing caries in children. Professionally applied fluoride treatments include varnishes and gels such as sodium fluoride(NaF), difluorosilane, and acidulated phosphate fluoride, while self-applied fluoride products, such as dentifrices, gels, and rinses, are widely available [15]. Sodium monofluorophosphate (SmFP), a commonly used fluoridated dentifrice, is synthesized from sodium met phosphate and sodium fluoride. Upon dissolution in saliva, SmFP releases monofluorophosphate complexes that facilitate fluoride ion integration into hydroxyapatite crystals, forming Fluor apatite and enhancing remineralization [16 - 19].

Despite its efficacy, fluoride dentifrices pose risks for young children, particularly those under six years of age, who may accidentally ingest significant amounts during brushing. This raises the risk of dental fluorosis, characterized by enamel discoloration, and acute fluoride toxicity, which can cause nausea, vomiting, and severe systemic complications in extreme cases [20, 21]. These concerns have driven research into safer, non-fluoridated demineralizing agents.

One such alternative is Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), derived from milk proteins. CPP-ACP mimics salivary processes by releasing Ca and PO_3 ions that facilitate remineralization of early enamel caries [22]. However, its milk-derived nature poses allergenic risks, including severe IgE-mediated reactions such as anaphylaxis, particularly in children with lactose intolerance [23].

four days (Figure 4b), the enamel surface exhibited saddle-shaped, beehive-like structures, reflecting significant demineralization. Post-remineralization, the enamel treated with artificial saliva (Figure 4c) showed a coating of remineralize material, leaving several irregularly shaped porosities. Enamel treated with sodium monofluorophosphate dentifrice (Figure 4d) displayed a dense, non-homogeneous cloudy coating with crater-like depressions.

Proanthocyanidins (PACs), bioactive phytochemicals abundant in grape seeds, exhibit potent antioxidant and antiquaries properties. These compounds interact with enamel proline, promoting mineral deposition and inhibiting bacterial adhesion. PACs also chelate Ca ions to form insoluble complexes and interact with collagen to enhance remineralization, particularly in acidic conditions [24]. Given the high concentration of PACs in red grape seeds (75–80%), a dentifrice containing pure proanthocyanidins was formulated and tested for its remineralization potential in this study.

The study used Vickers Hardness Testing (VHT) for quantitative analysis of enamel remineralization, offering greater precision compared to other methods. Scanning electron microscopy (SEM) provided qualitative insights into enamel surface morphology during demineralization and remineralization [25]. Demineralization was achieved using a solution of acetic acid (pH 4.5), a method adapted from established models, which closely mimics the conditions leading to early enamel caries [26-28].

The baseline mean Vickers Enamel Micro hardness (VEMH) values for primary teeth were consistent with prior studies, with post-demineralization values significantly reduced. After remineralization, the proanthocyanidin dentifrice demonstrated the highest VEMH, outperforming sodium monofluorophosphate, CPP-ACP, and artificial saliva. These findings align with earlier research highlighting the superior remineralization capabilities of proanthocyanidins compared to both fluoridated and non-fluoridated agents [29 - 34].

SEM analysis revealed distinct surface changes across groups. The proanthocyanidins group exhibited a dense, homogeneous layer with minimal porosity, indicative of effective extrafibrillar mineralization. This superior performance can be from PACs' ability to chelate calcium ions, inhibit matrix metalloproteinase, and form dense mineral deposits, preventing acid penetration and caries progression [35].

This study highlights the prospective of grape seed proanthocyanidins as a safe, natural, and cost-effective alternative to traditional fluoride and non-fluoride dentifrices. The proanthocyanidin

dentifrice demonstrated enhanced mechanical and ultra-structural remineralization properties. However, limitations include the absence of Energy dispersive X-ray analysis (EDAX) for mineral content quantification and the inherent inability of in vitro models to fully replicate complex oral conditions. In order to confirm these results and investigate the wider clinical usefulness of proanthocyanidins, future research should concentrate on in vivo investigations.

CONCLUSION

In conclusion, grape seed proanthocyanidin dentifrice proved more effective in enhancing enamel hardness and promoting extrafibrillar mineralization compared to fluoridated and non-fluoridated dentifrices. However, without mineral content analysis and given the limitations of in vitro testing, further clinical studies are required to validate these findings.

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